A Vitamin Digest

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Introduction

THE STORY told in the following pages is an outgrowth of an earlier and even more limited presentation, a *Lederle Laboratories Bulletin* issued in 1942. The many requests for this condensed account of the various vitamins made it necessary for two reprintings and provided the stimulus to bring the review up to date.

There are numerous textbooks and reviews covering some aspects of one or all of the vitamins but there is no available, condensed (or abridged) story which provides the reader with a good general understanding of the subject.

An attempt has been made to trace briefly the development of our knowledge in this field—a story that has been unfolding for about 40 years. The reader must be impressed by the use made of small animals, and more recently by bacteria as aids in isolating, identifying and assaying the respective vitamins. Considerable space has been used to show what happens to the various vitamins during handling, storage, cooking, etc. Through our ability to produce vitamin deficiencies in animals we have been able to show how several vitamins play an important rôle in metabolizing foods and maintaining normal functions of various organs and structures. It is very difficult to keep information on the various vitamins up to date. During the months in which the manuscript was in preparation several developments necessitated changes. During the next few years we are sure to witness the addition of many new facts—this is especially true in the case of folic acid and vitamin B_{12}.

Acknowledgments

In assembling the data presented in this review, the Author has made extensive use of the scientific literature as well as the various books dealing with the several vitamins.

While space does not permit specific accounting for each source of information, the writer hereby makes a general acknowledgment.

Miss Lillian Grosulak and Library Staff deserve special mention for the careful checking of references.

G. W. C.

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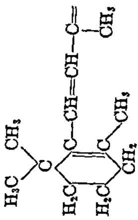
Chapter 1

Provitamins A—Vitamin A

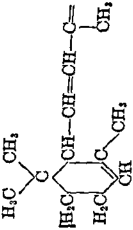
ALTHOUGH most of our knowledge of the various vitamins and their effects upon man and animals has been gained during the past 50 years, provitamin A (carotene) was isolated from carrots in 1831. It was 90 years later when the vitamin A activity of the carotenoids was discovered! The work of McCollum and Davis[1,2] and that of Osborne and Mendel[3,4] (1913–1915) demonstrated that a fat-soluble factor was necessary for growing animals and that the substance was present in butter fat and cod liver oil.

Two vitamins A and several provitamins A (carotenes) are known. The provitamins A, found abundantly in plants together with chlorophyll, and possessing A activity for man and animals, belong to a class of organic compounds containing both aliphatic and aromatic components and are commonly known as the carotenoids.

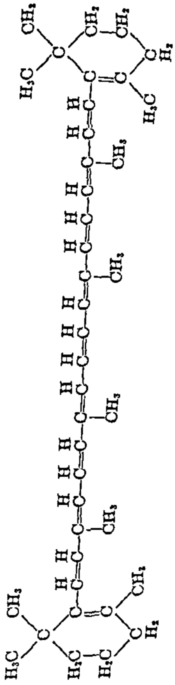
Chemically, the carotenes (polyenes) are characterized by a long aliphatic chain with a definite pattern of double bonds (—C═C—C═) which is responsible for the color exhibited by the group.[5] The aliphatic chain of 18 carbons, with the pattern of double bonds and 4 methyl groups as side chains, is the same for all of the provitamins A, the carotenes differing from each other by the structure of the aromatic groups at each end of the aliphatic chain. In beta-carotene the two groups are the same, beta-ionone, and it is commonly referred to as beta,beta-carotene. The alpha-carotene has an alpha-ionone ring and a beta-ionone ring and is commonly called alpha,beta-carotene. The gamma-carotene has one beta-ionone ring and the same number of carbon atoms but no ionone ring (pseudo-ionone structure) on the other end of the aliphatic chain and is known as gamma,beta-carotene. Although the hydrolysis of beta-carotene theoretically should



Beta-Ionone



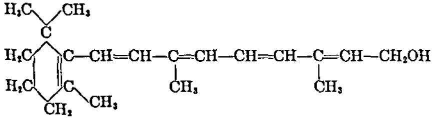
Alpha-Ionone



Beta-Carotene

Crystalline vitamin A, from propylene glycol or ethyl formate, melts at 63–64 °C.[8] Vitamin A (alcohol) distills at 120 to 125 °C. at 5 x 10^{-3} mm. and natural A esters distill at 200 to 240 °C. at 10^{-3} mm. The ultraviolet absorption spectrum of vitamin A shows a sharp band at 328 $m\mu$.[9] The extinction coefficient

$E^{1\%}_{1cm}$ = 1725. According to Baxter, et al.,[10] crystalline vitamin A contains 4,500,000 U.S.P. (International) units per gram. Vitamin A is optically inactive. Solutions in oil are relatively stable, the esters of the vitamin being more readily stabilized than the free vitamin (alcohol). The carotenes and vitamin A are soluble in common fat solvents. Although heat-stable in an inert atmosphere (carbon dioxide, nitrogen) the vitamin is readily oxidized, oxidation results in biologically inactive decomposition products: (1) a substance showing an absorption maximum at 312 $m\mu$; (2) a substance exhibiting a maximum of 290 $m\mu$, accompanied by disappearance of the characteristic absorption band at 325 to 328 $m\mu$. Dann[11] found that the photochemical reaction caused by exposure to wave length 2650 Å more rapidly destroyed vitamin A than beta-carotene.



Vitamin A (Alcohol)

Vitamin A may be determined by chemical, physical and biological methods of assay. Although several different color reactions have been proposed, the Carr-Price[12] procedure is the one in general use. This test depends upon the formation of blue color when a solution of antimony trichloride is added to a chloroform solution of the vitamin. The color intensity may be determined by means of a colorimeter or a spectrophotometer. In general, the values obtained in fish liver oils by the Carr-Price method agree quite well with those obtained from biological assays. Vitamin A may be determined quantitatively by the spectrophotometer utilizing the extinction coefficient of the characteristic absorption band (325 to 328 $m\mu$).[13,14] The biological method, utilizing growth effects on young rats, continues to be an official U.S.P. assay procedure[15] and is the method which most closely indicates availability to man and animals.

stance known as vitamin A_2. It has not been isolated in pure form, nor can it be completely separated from vitamin A by any known means, including chromatographic adsorption or vacuum distillation. The ultraviolet absorption spectrum (in alcohol) for A_2 shows a maximum of 345 to 350 $m\mu$. It has been shown that A_2 has six conjugated ethylene linkages whereas vitamin A has only five. In the ultraviolet the reaction products of A and A_2 show the same absorption bands. From all evidence at hand it would appear that A_2 is the C_{22} homolog of vitamin A.[16] In support of the chemical data indicating close relationship between A and A_2 Morton and Creed[17] fed carotene to perch and dace and were able to demonstrate the presence of both A and A_2 in the fish livers. Although A_2 may have a biological value equal to that of vitamin A, *there is no present indication that it is important in animal nutrition.* There is some evidence to indicate the existence of a third vitamin (A_3).[9,18]

Animals usually ingest more provitamins A than are required but apparently under normal conditions the conversion is not quantitative. According to Baumann, Riising and Steenbock[19] animals may convert 70 to 80 per cent of a limited provitamin intake into vitamin A. The facts at hand indicate the presence of a special regulatory mechanism which converts the provitamins A to vitamin A according to body needs. Animals differ considerably in the ability to convert provitamins into vitamin A. Rats, chickens,[20] guinea pigs,[21] rabbits,[21] pigs[22] and cattle[23] have the capacity to make the conversion (species vary in efficiency) but carnivorous animals (cats, foxes)[24] apparently depend on vitamin A in the food. Koehn[25] carried out carefully controlled experiments to determine the relative biological activity of beta-carotene, vitamin A alcohol and acetate, for the rat. Under conditions of the experiments, highly purified diets plus adequate amounts of all known dietary essentials including alpha-tocopherol, the data showed that the rat quantitatively converted the beta-carotene into vitamin A. Hickman,[26] though not agreeing with Koehn concerning the quantitative conversion of carotene, also believes that the variable results frequently obtained in the U.S.P. assays for A activity are due to varying and inadequate amounts of alpha-tocopherol in the diet.

It is estimated that vegetables and fruits supply approximately 65 per cent of the vitamin A present in the U.S. dietary and about half of the total vitamin A is provided by the leafy green and yellow vegetables (including sweet potatoes).[27] However, it has been shown by Graves[28] that the chemical estimation of carotene is a poor guide to the use of carotene-containing vegetables in nutritional studies. It has been shown also that the same amount of carotene derived from different vegetables, caused marked variations in growth and liver storage of vitamin A and that the foodstuffs rich in yellow pigments were least efficient in promoting vitamin A storage.[29] Callison and Orent-Keiles[30] found, by human dietary experiments, that carotene from different sources exhibits great variation in its availability to man, and other human experiments indicate as much as 75 per cent of carotene from carrots may be excreted in the feces.[23] Using the rat growth bioassay, Callison et al.[31] found that the rat utilized only 34 to 41 per cent of the carotene present in carrots and not more than 37 per cent of the provitamins present in sweet potatoes. Using a modified method for estimating the carotene content of vegetable matter, Zimmerman, Tressler and Maynard[32] showed that much of the yellow pigment in sweet corn is a carotenoid, cryptoxanthin, possessing a low degree of vitamin A activity, about half that of beta-carotene. This and other similar work suggests that a conversion factor, based on biologic assay, is necessary for each carotene-bearing foodstuff used in nutritional studies.

Certain pathological conditions such as diabetes,[33,34] phosphorus poisoning of the liver,[35] adversely affect the conversion of provitamins A into vitamin A. Brazer and Curtis[34] found that 60,000 U.S.P. units of crystalline carotene, dissolved in a vegetable oil, given daily for 14 days to juvenile diabetes mellitus patients did not affect the poor light adaptation of these individuals whereas the same number of units of vitamin A, derived from fish liver oil concentrates, caused a prompt return to normal (3 to 21 days). If one determines the levels of carotene and vitamin A in the blood, it is possible to follow the conversion of provitamins to vitamin A. It has been observed that each person usually maintains a characteristic definite level of vitamin A in the blood whereas the carotene blood level depends upon the intake. Dur-

ing depletion periods, animals tend to maintain essentially normal blood levels of vitamin A until all stores of A and provitamin A are converted whereas during the same interval, there has been progressive decline in the carotene level.[36]

Because of the marked ability of different species of fish to store vitamin A in the liver, fish liver oils are the richest natural source of this vitamin. There is a marked difference in the A storage from specie to specie and differences in the same specie due to season of the year, food sources, etc. Average cod liver oil contains 600 to 800 U.S.P. Units, Boston mackerel may contain up to 30,000 units per gram, bluefin and yellowfin tuna 60 to 70,000 units per gram, swordfish 250,000 units per gram and black seabass up to 600,000 units per gram.

The importance of vitamin A as a food supplement and its extensive use as a therapeutic agent have resulted in a great many methods for the production of palatable, stable, vitamin A concentrates. Although crystalline vitamin A is an article of commerce, it has not replaced the fish liver oil concentrates which may be prepared by any one of the three commonly used commercial methods:

(1) The oldest of these employs alkali saponification of the oil and extraction of the non-saponifiable fraction by some suitable solvent;

(2) In another method, saponification is also the first step but, instead of extracting with a solvent, the nonsaponifiable fraction, after removal of the sterols by chilling, is subjected to molecular distillation. The distillate containing the free vitamin A alcohol may contain 1,000,000 to 2,000,000 U.S.P. units per gram;

(3) By the third method (of increasing commercial importance) the crude fish liver oils are subjected directly to molecular distillation.[37] By molecular distillation of cod liver oil it is possible to obtain two different fractions coming off at widely different temperatures which represent quantitatively the total vitamin A activity of the oil. This suggests two forms of the vitamin and since the high boiling fraction can be saponified and converted to the low boiling fraction, it becomes apparent that cod liver oil contains free vitamin A (alcohol) and vitamin A esters.[38] The A potency of the distillates may be as high as 500,000 units per gram.

This process also makes it possible to use oils with a naturally low vitamin A content. Tischer[39] identified one of the vitamin A esters, present in cod liver oil, as vitamin A palmitate.

The exact mechanisms through which vitamin A contributes to the physiological processes of man and animals are unknown. It is likewise not known whether or not vitamin A *per se* acts in the animal organism but since there is always some free vitamin *A* to be found in the tissues, it has been assumed that it is the active molecule and since the vitamin is stored as various esters (both saturated and unsaturated fatty acids) in the liver, it is likewise assumed that esterification is principally for the purpose of storage. Provitamins A and vitamin A are readily absorbed from the gastrointestinal tract. Some absorption takes place through the skin[40] and they are also utilized when injected subcutaneously or intramuscularly. Under normal conditions both provitamins A and vitamin A are rapidly absorbed from the intestinal tract; in man the peak with vitamin A is reached in three to five hours, with the provitamin A, the peak may not be reached until the 7th or 8th hour. Absorption from the gut is accompanied by increased blood levels.[41] Absorption from the intestinal tract depends upon the nature of the fats present—the more unsaturated the oil, the better the absorption. In addition to the presence of fats it is generally held that the absorption of vitamin A also depends upon the presence of bile acids and probably pancreatic lipases. Greaves and Schmidt[35] found that carotene was not absorbed in presence of jaundice or in phosphorus treated rats. Adlersberg et al.[42] studied the absorption of vitamin A in liver disease and sprue. The patients studied included 18 normal controls, 14 patients with liver disease and two patients with sprue. The data showed that in severe hepatic damage or complete biliary obstruction (characterized by flat vitamin A curves in the tolerance test), bile acids had no effect upon the shape of the curve; in milder liver disease the bile acids moderately accelerated intestinal absorption. In cases of hepatic damage, lecithin had no effect on absorption of vitamin A whereas in sprue, lecithin did improve absorption.

Gray et al.[43] showed that the esters of vitamin A are hydrolysed prior to absorption, and Drummond[44] and co-workers demon-

strated that reesterification occurs soon after or during absorption. The work of Mattson[45] indicates that in the rat the conversion of provitamin A (carotene) takes place in the intestinal wall. There is an apparently normal blood level of vitamin A which is independent of the amount of vitamin stored and which is subject to temporary increases during the period of absorption. When the concentration of vitamin A in the plasma reaches approximately 1 unit per ml, there is no further increase even though excessive amounts of vitamin A are ingested. In A-depleted rats there is no detectable amount of the vitamin in the blood plasma. On a low vitamin A (or provitamin A) intake no storage takes place in the liver, but when the intake is more than the body needs there is a rapid deposition of vitamin A in the liver.[46] With the ingestion of excessive amounts of the vitamin there is less efficient storage.

Since one of the principal actions of vitamin A is to stimulate the building of new cells, it is to be expected that a deficient intake would result in a decreased growth rate of the young. Prominent in the A-deficiency syndrome is the atrophy of the epithelium followed by the substitution of stratified, keratinized cells for the normal epithelial structures.[47] These structural changes are observed early in the mucosa of the mouth and respiratory tract. During the course of a long, inadequate intake of vitamin A, the epithelium of the eyes, the ductless glands, the urethra, kidney and intestinal tract are also involved. The vaginal epithelium is very sensitive to a lack of vitamin A and senile vaginitis is frequently observed.[48] In man there is a generalized dryness of the skin, the hair appears very dry and there may be extensive hyperkeratosis of the hair follicles. The cutaneous eruptions involve first the arms and thighs, and may extend to all parts of the body.[49] Warkany and Roth[50,51] observed soft tissue abnormalities in A-deficient rats and, as the carotene in the diet was reduced, there was a lowered fertility and a marked increase in the per cent of abnormal young. It has been repeatedly shown that the sex organs of rats degenerate when the animals are long maintained on a diet deficient in vitamin A. The studies on hypovitaminosis A by Chieffi and Kirk,[52] comprising 106 individuals belonging in the middle age and old person groups, showed a high incidence

of hyperkeratosis of skin follicles and localized conjunctival thickening in all persons exhibiting 1–15 μg. per cent plasma levels of vitamin A. Dry skin and blepharoconjuctivitis were observed more often in those showing low plasma levels. Jeghers[53] surveyed the health and dietary habits of a large number (160) of medical students and found that 35 per cent had poor dark adaptation and 12 per cent had clinical manifestations of vitamin A deficiency (dry skin, dry conjunctivae, blepharitis and follicular hyperkeratosis). Jeghers decided that poor selection of foods and skipping of meals were the main cause of the deficiency and noted that impaired dark adaptation preceded the epithelial changes.

In combination with a specific protein, vitamin A is necessary for the activity of visual purple. Rhodopsin, visual purple, is an albumin-carotenoid with an absorption maximum of 500 $m\mu$. Retinene, the prosthetic group, is of unknown composition[54,55] but is related to the carotenes. The pigmented layers of the eye contain relatively large amounts of vitamin A and supply the vitamin A required for the regeneration of visual purple. The decreased ability for dark adaptation (night blindness, nyctalopia) is considered as possibly the earliest sign of vitamin A deficiency.[56]

Vitamin A and provitamin A are secreted in the milk. In cows the ratio of A to provitamin varies considerably with the breed and is somewhat influenced by the feed although the total vitamin A activity of milk is quite constant, irrespective of breed.[57] Human milk contains much more vitamin A activity (5 to 10 times) than cow's milk. The vitamin A content of the body tissues of all newborn animals is low but no symptoms of A-deficiency are observed if the mothers have had an adequate intake. The presence of vitamin A in the newborn implies that there is some special mechanism which permits the transfer of vitamin A through the placental wall. After birth, there is a rapid increase in the A content of the tissues and within a few weeks reaches the normal adult level.

Brown and Morgan[58] state: "Vitamin A may therefore be considered essential for the growth of tissue protein but not for its maintenance." In a subsequent report[59] these investigators were

unable to demonstrate, as shown by nitrogen exchange, a relationship between total metabolism and vitamin A requirements. In a recent editorial[60] discussion of the thyroid—vitamin A relationship, it is pointed out that hypothyroid patients lack the ability to convert provitamin A (carotene) to vitamin A and that rats receiving thiouracil fail to show any storage of vitamin A in the liver and exhibit signs of vitamin A deficiency even though an adequate supply of carotene is present. Thiouracil animals are able to absorb and utilize vitamin A *per se*.

It has been shown that large doses of vitamin A in the form of certain vitamin A concentrates[61,62] or natural fish liver oils[63] (notably jewfish oil) produce toxic effects when given to experimental animals. Some lots of jewfish oil were found to be more toxic than others but there appeared to be no relationship between vitamin A content and toxic properties. Vedder and Rosenberg[63] also found that in molecular distillation the toxic substance remained in the still. These investigators gave young rats (50 grams weight) 100,000 U.S.P. units of vitamin A, supplied by fish oil concentrates, daily for 100 days (a total of 10,000,000 units) without observing definite toxic symptoms. During this period of intensive vitamin intake, the maximum storage in the liver (rats) did not exceed 5,000 U.S.P. units per gram. While it is possible to produce hypervitaminosis A in rats, it requires a gross excess of the vitamin.

The question of requirements involves two standards, one for the provitamins A, and one for vitamin A. The U.S.P. (International) units of carotene and vitamin A are equal only under the conditions specified in the test, they may not be equal in the normally growing animal.[64] Evidence indicates that the general requirements for vitamin A may be correlated with body weight and on this basis it has been shown that man, horse, dog, cattle, sheep, pigs, rabbits, rats, and other animals[64,65,66,67,68,69] require about the same amount: 40 U.S.P. units of beta-carotene or 20 U.S.P. units of vitamin A per kilo of body weight.[64,69] Although these amounts provide the minimum requirement for normal growth and prevent the development of deficiency symptoms, there is little or no storage. For maximum nutritional effects, one should allow about three times the minimum vitamin A or provitamin A requirements.

denum, upper and lower jejunum and the ileum were fixed in formalin. Subsequently frozen sections of these tissues were studied and, based on vitamin A fluorescence, it was found that about three times as much A was absorbed from the aqueous as from the oily medium. Dispersion of the vitamin into fine particles also accelerated passage through the intestinal wall. Based on a study of blood levels of vitamin A, Sobel et al.[76] also showed that the vitamin was more rapidly absorbed from aqueous media. Other experiments, in which absorption was estimated from liver storage,[77] showed rats stored about three times as much from an aqueous medium as when the same vitamin concentrate was dispersed in maize oil.[76] With distilled vitamin esters, tested in the same manner, there was about twice as much liver storage when the esters were dispersed in water as when given in oil. This difference in absorption from aqueous and oily media was also observed in children with impaired absorption (celiac syndrome).[36,78] With[79] showed that when carotene or vitamin A is dispersed in mineral oil and administered to rats there is a marked drop in absorption and storage, being not more than 50 per cent of the value obtained when the vitamin is given in sesame or peanut oil. However, except for the daily ingestion of large amounts of mineral oil, it is doubtful whether this finding plays an important role in human nutrition.

Since man and animals in general do not possess the ability to synthesize the vitamin, it must be supplied in the foods ingested. Vitamin A and the provitamin A, the carotenes, are widely distributed in nature; vitamin A *per se* is found in whole milk, cream, butter, yolk of egg, mammalian liver and especially in fish liver oils; the carotenes are distributed abundantly in green leafy vegetables (lettuce, chard, spinach) and in many of the yellow vegetables (carrots, sweet potatoes, rutabagas). From a nutritional standpoint, alpha, beta and gamma-carotenes are the important precursors of vitamin A. There are other highly colored pigments present in plants (lycopene, the red pigment present in ripe tomatoes; xanthophyll, the yellow lipochrome of leaves; capsathene, in red peppers) which do not belong to the provitamin A group.

The common use of butter by the average American family is

adequate reason to consider variations in the vitamin A activity of this product. Lord[80] made a two year (Aug. 1941 to Aug. 1943) study on 70 pure-bread Ayrshire cows, of various ages, and in different stages of lactation. Over the entire period the butter produced by this herd averaged 31 (International) U.S.P. units per gram when pasture fed and 15 (International) U.S.P. units when they were stall-fed. In these studies carotene contributed 20 to 28 per cent of the vitamin A activity of the butter. Berl and Peterson[81] made an extensive study of Wisconsin butter and found that summer butter (July–Sept.) averaged 18,000 (International) U.S.P. units per pound; January butter averaged 10,500 units and March butter averaged only 9,500 units per pound. Studying the effects of the breed of cattle, these investigators found that September butter from Guernsey milk contained 9.47 μg. of beta-carotene per gram and 7.34 μg. of vitamin A per gram. During the same period Holstein milk contained 5.66 μg. of beta-carotene per gram and 10.39 μg. of vitamin A per gram. Ellenberger et al.[82] examined 150 samples of 5 nationally advertised brands of butter and after noting the well recognized seasonal variations (lowest February to May, highest point in October) stated that there was no significant loss of vitamin activity during the period normally required for the product to pass from manufacturer to consumer. The average vitamin A potency for a year was 15,640 (International) U.S.P. units per pound, beta-carotene contributing approximately 21 per cent and vitamin A 79 per cent of this value.

The official U.S.P. method[15] for determining the potency of vitamin A preparations uses the rat growth (weight and ophthalmia) method. The details as to this assay need not be set forth here; suffice it to say that it requires the breeding of test animals, the preparation and use of special diets and finally a comparison of the unknown with a standard reference oil. From the time the animals are ready to be put on the depletion diet until the assay is completed, a minimum of seven weeks is required. One U.S.P. unit of vitamin A is equal in growth-promoting and antiophthalmic activities for the rat, to one International Unit of vitamin A (0.6 μg. of beta-carotene) as defined and adopted by the Second International Conference on Vitamin Standardization in 1934.

The list of foodstuffs providing the *provitamins* A and/or vitamin A is too long to include in this presentation. The following foodstuffs, all readily available, rank very high in vitamin A activity: lettuce, spinach, chard, escarole, butter, cheese, eggs, liver, milk,* green peas, green peppers, carrots, sweet potatoes, apricots and peaches.[83,84] The more liberal and optimal amounts of A (National Research Council)[85] needed to meet the demands of various age groups and different physiological states are shown in detail in Table II, page 242.

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Chapter 2

Provitamins D and Vitamins D

RICKETS has been recognized for more than 125 years.
were also inferences as to the curative effects of sunlight pr
the work of Palm[1] who associated the incidence of rickets v
deficiency of sunlight. In 1906 Hopkins[2a,2b] indicated that r
might be caused by the absence of "an accessory foodstuff.'
1919 Mellanby[3] produced rickets in puppies and also disco
that certain animal fats cured the animals, demonstrating fo
first time the nutritional importance of animal fats and th
played in the calcification of bones. In 1920–21 McCo
Simmonds, et al.,[4] also Sherman and Pappenheimer[5a,5b] usec
trolled diets to produce rickets in rats and, in subsequent p
McCollum et al.[6a,6b] *proved by experimental means the diff*
properties possessed by the antirachitic factor and vitami
About the same time Hume,[7] Sheets and Funk[8] (working
pendently) reported that the A-deficiency syndrome was
cured by light. Hess and co-workers[9,10] confirmed the ea
work of Huldschinsky[11] and others, proved that sunlight c
human rickets, and demonstrated that cod liver oil and sun
possessed identical curative effects in human rickets and also
syndrome produced experimentally in animals (rats).

Although several sterol compounds have been listed under
vitamins D, *actually there are only two of importance:* (1) er
terol, present in plants, molds and yeast; (2) 7-dehydro-ch
terol, present in the tissues of man and animals. The provitai
may be activated to vitamins D by a number of procedures
under a variety of conditions, in brief all involve an inpu
energy into the provitamin molecule.

Bills et al.[12,13] and others[14,15,16,17] have shown that the conver
of the provitamins is not a single, simple reaction but a serie
reactions in which several substances are formed before vitami

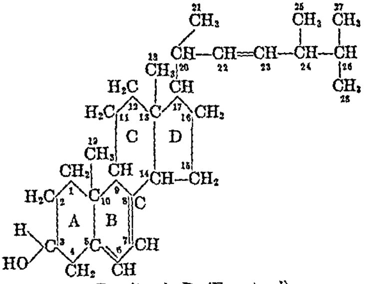
is obtained. Though the intermediate products may differ, depending upon the procedure and conditions employed, it seems certain that the vitamin D obtained is identical by all methods. There is no equilibrium between the irradiation products so the photochemical reaction is reversible. Briefly the course of reactions[16] is as follows: ergosterol → lumisterol$_2$ → pro-tachysterol$_2$ → tachysterol$_2$ → vitamin D_2 ("Calciferol").

Continued irradiation of D_2 produces toxisterol$_2$, a very toxic compound, and two end-products; suprasterols$_2$ I and II. Of the two series of compounds only vitamin D_2 and D_3 possess marked antirachitic activity, the intermediates are inactive. Dihydrotachysterol$_2$ (antitetany compound No. 10, "A.T.10"), prepared from tachysterol by reduction, possess slight antirachitic properties,[18] but more important, it causes an increase in the calcium content of the blood, and is used clinically to treat idiopathic and post-operative tetany[19,20,21] (hypoparathyroid).

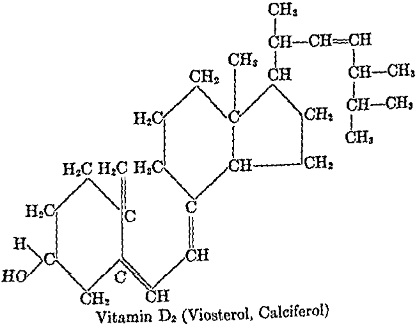
Although Hickman et al.[22] have demonstrated that six D vitamins may be obtained from cod liver oil by molecular distillation, only four vitamins D (D_2, D_3, D_4, D_5) have been prepared in essentially pure form. Of the six D vitamins present in cod liver oil, D_2 and D_3 constitute the major portion, and two are present in traces only. Only two vitamins D (D_2 and D_3) have been isolated in a relatively pure form from fish liver oils.[23] The diverse nature of the D vitamins found in fish liver oils has been repeatedly demonstrated on different animal species. While the rat has always been used to establish the vitamin D potency of various substances used as foods or dietary supplements, it has been shown that the chick is a much more sensitive test animal. The chick response to one "rat unit" of D, contained in U.S.P. Reference Cod Liver Oil is arbitrarily designated as one "chick unit" and equals 100 per cent activity. When compared in this manner it was found that vitamin D_3 was 100 per cent chick active but D_2 possessed almost no chick activity. Bills et al.[24] examined a large number of fish liver oils for chick potency and found some that were 200 to 300 per cent as active as the Reference cod liver oil. Jukes and Sanford[25] showed that the "chick unit" of vitamin D may not constitute a "turkey unit."

In the pure state, each of the known vitamins D is obtained as

white, odorless crystals. They are readily soluble in fats and in the common fat solvents (ether, chloroform, acetone, etc.) and exhibit a characteristic absorption spectrum with one maximum at 265 mμ. Crystalline vitamin D_2 (Calciferol) melts at 115 to 117°C., is thermolabile but may be kept for many months if stored at low temperature (2°C.) and protected from light and oxygen (air).



Provitamin D_2 (Ergosterol)



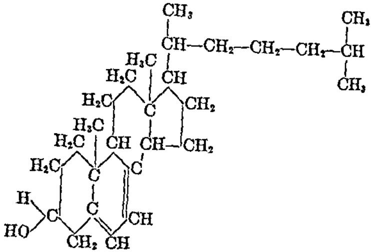
Vitamin D_2 (Viosterol, Calciferol)

The compound is optically active, $[\alpha]_D^{20} = +103°$ in absolute alcohol, +82.6° in acetone, +91.2° in ether. One mg. = 40,000 U.S.P. (International) units. Crystalline D_3 melts at 82 to 83°C. The compound is optically active, $[\alpha]_D^{20} = +83.3°$ in acetone.

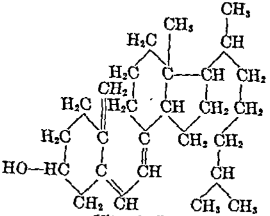
Although several theories have been advanced to account for the large amounts of vitamin D stored in the liver oils of various species of fish,[26,27,28] none, however, is supported by adequate evidence.

The widespread occurrence of rickets indicates that man is unable to synthesize appreciable amounts of vitamin D. It has been shown that the skin of man[29,30] and animals (cattle, pigs)[29,31] contains relatively large amounts of provitamin D, and it is likewise certain that man and animals acquire considerable quantities of vitamin D through exposure of the skin to ultra-violet light. The amount of vitamin D thus formed is subject to seasonal variations, being greatest during the summer months. This effect of decreased sunshine is reflected in the lowered vitamin D content of milk[32] and eggs[33] during the winter months.

Drake, Tisdall and Brown[34] compared the antirachitic effect of irradiated cholesterol and cod liver oil, administering each to approximately 75 infants. Both agents were effective prophylactic agents when given in daily doses of 150 International units. The daily intake of 300 to 750 International units, in the form of irradiated cholesterol, cured infants showing moderate to severe degrees of rickets. Subsequently, Lewis[35] administered varying doses (135 to 870 U.S.P. units per day) of cod liver oil, viosterol or percomorph liver oil to large groups of infants throughout an Eastern winter season. With a daily prophylactic dose (135 to 870 U.S.P. units) all of the 170 infants were well protected—only three infants on the 135 units per day developed evidences of rickets. This suggested that the three substances possessed equivalent antirachitic properties (for man). Only 14 infants received curative treatment. Thus the comparison of the relative effectiveness was less significant, but did suggest that on an equal unit basis viosterol was somewhat less effective as a curative agent. Hess et al.[36] carried out extensive studies to evaluate the antirachitic agents in both rat and clinical units. This work contributed greatly to the proper use of these agents.



Provitamin D_3 (7-Dehydro-Cholesterol)



Vitamin D_3

The principal absorption of vitamin D takes place in the small intestine, the process is facilitated by the presence of moderate amounts of fat[37] (not from mineral oil)[38a,38b] and bile.[39] The vitamin passes from the intestinal tract to the circulating blood, lymph, tissue fluids and to some extent to all parts of the body. Normal human blood serum contains 50 to 135 U.S.P. units of vitamin D per 100 cc.

Although man and all animals studied do not have any special storage organ, certain tissues, especially those rich in lipids, (brain, liver, spleen) contain substantial amounts.[40] Even though the mothers received an abundant supply of D during gestation, the newborn of man and animals have very little vitamin D in their tissues. The liver and in a lesser degree the viscera are special storage organs for fish only.

Vitamins D_2 and D_3 are readily metabolized as evident by the prompt appearance of the vitamin in the milk of all mammals and the eggs of fowl. If D_2 is fed, D_2 appears in the milk or in the eggs: if D_3 is ingested then it is found in milk[41] and in eggs.[42] Eggs laid by hens on a diet deficient in D are low in D and do not hatch well. The vitamins D from fish liver oils, D_2 from activated ergosterol, D_3 from activated 7-dehydro-cholesterol, and activated "mussel provitamin" are all metabolized but in varying degrees of efficiency. It is known that vitamin D is excreted[43] principally via the bile into the intestinal tract and that some of the vitamin is destroyed, however there are no available data to indicate the amount of D actually destroyed. No vitamin D appears in the urine.

As indicated in sections covering other vitamins, a lack of any one of these accessory substances retards growth, in some instances this may be a secondary effect. However, in case of vitamin D, the growth-promoting effect is a primary action.[44] The gross effect of vitamin D deficiency is obviously a severe disturbance of mineral metabolism resulting in a syndrome known as "rickets." *Excluding cases of frank tetany, with low* serum calcium, Howland and Kramer[45a5b] found many rachitic children with essentially normal serum calcium; however, all cases of active rickets showed lowered values, often extreme for serum inorganic phosphorus. Soon after Mellanby[3] produced experimental rickets in dogs, two groups[4,5a,5b] working independently produced experimental rickets in rats. Generally speaking the rat has a low requirement for vitamin D but the rat syndrome, which resembles the human disease, can be produced by a special diet which contains an abnormal proportion of calcium and phosphorus. A diet low in calcium and high in phosphorus, or a diet high in calcium and low in phosphorus may be used. Either the Steenbock-Black[27] diet No. 2965 or the McCollum-Simmonds[6a] Diet No. 3143 gives good results. In each the ratio of calcium to phosphorus is approximately 5 to 1.

In the growth of bones three different types of tissue are involved: cartilage → osteoblasts → bone cells. In the early stages of vitamin D deficiency the osteoblasts cease forming, cartilage continues to form and uncalcified osteoid tissue accumulates.

The administration of vitamin D causes a complete change (apparent in 24 hours) in the course of events: blood vessels penetrate the unorganized cartilage cells; osteoblasts appear in orderly arrangement; and calcification begins and normal bone is formed.

Numerous and various experiments indicate that as far as man is concerned the formation of normal bone is dependent upon the presence of vitamin D *per se* rather than to any effect on the absorption of calcium and phosphorus. According to Benjamin and Hess[46a,46b] the blood serum calcium exists in four different forms; one of the two diffusible forms is made up of a calcium-phosphorus complex and is the postulated precursor of the bone salts. The amount of this complex present in the blood serum is apparently independent of either the calcium or phosphorus content and while the total calcium of the blood may not be noticeably affected by a rachitic condition, there are significant changes in the amounts of the various forms. The metabolism of phosphorus is quite different: the inorganic phosphate and the phosphate esters are constantly and reversibly changing. Vitamin D deficiency results in a marked drop in serum phosphorus, from a normal of 4 to 6 mg. per 100 cc. to 1 to 2 mg. Excretion of phosphorus continues due to the removal of phosphorus from the bones and there is a marked increase in the blood serum content of the phosphotase enzyme.[47a,47b] The altered metabolism of calcium and phosphorus (the lack of calcification) is especially noticeable at the epiphyses, the points of active growth. As a result the ends of the long bones become greatly enlarged due to the excessive formation of cartilage. Enlargements of the junctions of the rib occur ("rachitic rosary"). In severe rickets there may be malformation of the skull, the jaw is poorly shaped, eruption of the teeth is delayed and tooth enamel is poorly formed.[48] Curvature (spine, long bones) of the bones is seen in the late stages. The administration of vitamin D to a rachitic subject causes a rapid rise in serum phosphorus and frequently it may reach higher than normal values. Frequently a low blood calcium (7 to 8 mg. per 100 cc.), as in severe D deficiency, and the high phosphorus causes tetany, similar to that seen in hypoparathyroidism. As indicated earlier a compound closely related to vitamin D, dihydrotachysterol ("A.T. No. 10") symptomatically cures the

tetany by increasing the blood calcium.[13] Freudenberg and Welker[49] found that a lack of D affects carbohydrate metabolism by retarding phosphorylation.

The idea of using large single doses of vitamin D ("Stoss Therapy") apparently came into clinical use about 1936. Briefly the method consists in giving one enormous dose of D_2 (Calciferol) or D_3 equivalent to 600,000 U.S.P. (International) units. The procedure has been recommended for the treatment of severe rickets where there is risk of fracture and in severe cases associated with acute or chronic infection. Following the one big dose, healing can be detected in a few days (by radiographic studies) and the process continues without additional therapy. Rambar et al.[50] found the single dose, intensive therapy very effective and monthly doses of 100,000 U.S.P. units also gave good results in the treatment of rickets in infants. No toxic symptoms, either clinical or laboratory, were observed. Wolf[51] treated 75 infants during the third to fifth months of life with 600,000 U.S.P. units of D, divided into two oral doses. The 18 showing mild rickets healed within two to seven months and remained healed. Another group of 44 infants received single oral doses of 600,000 U.S.P. units, all infants thrived and there were no toxic manifestations. The medical literature contains several references to the use of "massive doses" of vitamin D in the treatment of arthritis and psoriasis. Klassen and Curtis[52] used intensive D therapy in the treatment of four cases of atrophic spondilitis and degenerative arthritis of the spine. The initial doses 200,000 U.S.P. units was increased every third day so that during the tenth, eleventh and twelfth days each patient received 1,000,000 U.S.P. units of D_2 (total of 5,600,000 units for 12 days). No gastrointestinal disturbances or other signs of toxicity were observed. The urinary excretion of calcium and phosphorus increased in all four patients. Snyder and Squires[53] observed 23 rigidly controlled patients and reported that during a period of two years the entire group of 23 patients had not responded to any form of therapy. When treated with large doses of vitamin D (Ertron), most of the patients showed marked and substained clinical improvement. No serious toxic effects were observed. Abrams and Bauer[54] treated 18 cases of rheumatoid arthritis with large doses of vita-

volved or the expense incurred. Steinberg[55] came to sion that any effects produced in chronic arthritis by ›ses of vitamin D were nonspecific. It was impossible e the laboratory studies of calcium and phosphorus a with any clinical improvement or aggravation of the int symptoms. Steinberg listed the toxic symptoms ht be expected and warned that clinical investigators *intensive D therapy should look for: nausea, vomiting,* arrhea, anorexia, headache, vertigo, muscle and joint uent urination, thirst.

ı et al.[56] gave 175,000 to 700,000 U.S.P. units of D to rats and showed that surprisingly little D was retained ıal body, from 1.7 to 5.7 per cent. Of the total amount he liver accounted for 9 to 22 per cent. Warkany[57] the blood serum of normal children contained 30 to 50 (U.S.P. units?) per 100 cc. In a subsequent paper, t al.[58] reported vitamin D serum levels in adult patients l after the ingestion of 50,000 to 500,000 U.S.P. units *Following the administration of 400,000 to 500,000* ·, the blood serum values increased to between 9,000) units. After abruptly stopping all D intake, it re- e to six months for the D content of the blood to fall ıl level. No serious toxic symptoms were observed in riments.

ınd Stevenson[59] studied the course of healing in twelve ıildren; six received 2,000 units of D_2 daily and six .000 units of D_3 daily. From radiographic studies of ınd from the plasma phosphate values, it was concluded ıerapeutic effects of D_2 and D_3 were equal in the group *Iypervitaminosis D may occur in man or animals after* . It is therefore important to know the various signs ›ms of vitamin D toxicity. In man digestive disorders ; appetite declines; nausea, vomiting and diarrhea are observed. Laboratory findings may show an exten- on of serum calcium, up to 17 mg. per 100 cc., and as a

result there may be metastatic calcification in various tissues, especially in the kidneys, lungs, heart and blood vessels. Initially there is a period of calcium retention, later there may be extensive decalcification. Morgan et al.[60] found that the general toxicity of D_2 was greater than that of D_3 for dogs.

Remp and Marshall[61] determined by rat assays that crystalline D_2 and crystalline D_3 had equivalent potency, 40,000 U.S.P. units per mg. Correll and Wise[62] found that cod liver oil was more efficient than D_2 (irradiated ergosterol) in reducing the phosphate activity in the growing chick. Gerstenberger et al.[63] studied the effects of identical doses of D_2 and D_3 in monkeys. The data were definitely in favor of vitamin D_3. Coward[64] distributed separate solutions of D_2 and D_3 to nine collaborating groups. The data demonstrated that for the rat, D_2 and D_3 possess equivalent antirachitic activity, 40,000 U.S.P. (International) units per mg.

The vitamin D requirements of poultry have been studied extensively and it is customary to incorporate about 200 A.O.A.C. chick units per pound of feed for growing chicks and approximately 400 A.O.A.C. chick units per pound of ration for all laying stock.

Morgan[65] has shown that large breed dogs require much more vitamin D_2 than do the small breeds. The dog requirements for D_3 are much lower and more uniform.

Young pigs need vitamin D and unless the winter-time diet is adequate in this respect, there is a tendency to develop rickets.[66] For best growth it has been suggested to provide young pigs with about 100 U.S.P. units of D per kilo body weight. Sheep and young calves also benefit from an adequate amount of D in the feed, about 200 U.S.P. units per kilo for these species.

Jeans and Stearns[67a,67b] placed the vitamin D requirement of the full term artificially fed infant at 300 to 400 U.S.P. units per day. It seems probable that the premature infant may require twice as much D as the full term infant. Adolescents need vitamin D but Jeans and Stearns[67a] felt that the data were inadequate to permit estimation of exact requirements and suggested 300 to 400 U.S.P. units per day. No adult requirements were mentioned. Pregnant and lactating women require liberal amounts of vitamin D, at least 800 U.S.P. units per day. The usual daily dose for the

cure of rickets in infants is from 500 to 1500 U.S.P. units. Park[68] recommends 800 units of D per day during pregnancy and is emphatic in recommending continued attention to the D requirements of growing children and suggests that 400 U.S.P. units are adequate in most instances. Park also believes it is desirable to recommend vitamin D supplements for: (a) all who are deprived of sunshine (night workers, invalids); (b) those who have little or no milk in the diet; (c) persons suffering from fractures or bone operations; (d) pregnant women, irrespective of diet and season; (e) the aged who are confined; and (f) those who, for various reasons, have to observe dietary restrictions.

It is necessary to point out that vitamin D is not widely distributed in the foods generally consumed by man. The livers of edible animals (beef, pork, fowl) are undoubtedly the richest source of vitamin D. Butter, eggs and certain fish with high content of body fat are good sources of D. In connection with the D content of milk, Campion et al.[69] found that June milk from cows kept outdoors contained approximately three times as much D per quart, as the milk from cows kept indoors. Bechtel and Hoppert[70] found that summer milk contained eight times as much D as winter milk. Much of the milk supply of our cities is fortified so that one quart supplies 400 U.S.P. units, the daily requirement for a child.

The chick assay[71] method recommended by the Association of Official Agricultural Chemists is based upon ash content (in per cent) of the tibia, determined under standard conditions, "the A.O.A.C. Chick Unit." The chick potency of any given D preparation is determined by assaying the preparation on the basis of its rat unit value.

The rat assay has become a universally accepted procedure and is the method employed to establish the U.S.P.[72] (International unit). By this procedure groups of young rats are placed on a rachitogenic diet, either the Steenbock-Black diet or that of McCollum et al. may be used. Several groups of rats are fed the preparation to be tested; one or more groups receive the Standard Reference cod liver oil and one group is usually maintained on the special diet. The animals receive the D supplements for six days,

are sacrificed on the 8th day and the degree of rickets is estimated by one of the following procedures:

(1) The "Line Test"—this is a curative method;

(2) Ash content of the Bone—this is a prophylactic method;

(3) The radiographic method—examination of various bones for calcification; and

(4) the Body Weight method.

Two different groups (Nield, et al.,[73] Milas, et al.[74]) have proposed spectroscopic methods for estimating D_2 and D_3. There are no satisfactory chemical methods for the accurate determination of vitamin D activity.

Vitamin D is present in relatively few of the foodstuffs ordinarily appearing in the diet of man. The following indicates important dietary sources of vitamin D: Egg yolk, livers of edible animals, butter, a few fish with high content of body fat (salmon, herring) and fortified products (cereals, milk and margarines).

From the foregoing remarks it is evident that man is often dependent for a considerable part of his vitamin D requirement upon a certain amount of exposure to sunlight, during which time the ultraviolet rays transform the provitamin present in the skin into active vitamin D. The fortification of milk, margarine, bread and other cereals has made it much easier to obtain our daily requirement.

The more liberal and optimal amounts of D (National Research Council) needed to meet the demands of various age groups and different physiological states are shown in detail in Table II, page 242.

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Chapter 3

Tocopherols (Vitamin E)

IN 1920 Mattill and Conklin[1] noted poor reproduction in rats maintained on a special milk diet. During 1920–23 three widely separated laboratories[1,2a,2b,2c,3] published results of experimental work which indicated that under certain dietary regimens, which included ample quantities of protein, fat, carbohydrate and minerals as well as all of the known vitamins, young rats ceased to grow and lost the function of reproduction.

By the process of trial and elimination Evans and Bishop[2] and later Sure[3] demonstrated that sterility was not due to a lack of vitamins A, B, C and D, the mineral salts or the quality and quantity of protein in the diet, but was due to another vitamin, hitherto unrecognized (designated as "X"), for which was proposed the next letter in the vitamin alphabet, "E." In the period immediately following this work the terms "reproductive vitamin" and "antisterility vitamin" were freely used. The various symptoms observed in animals maintained on a vitamin E deficient diet leads one to regard this vitamin as an essential for the functioning of all body tissues. Retardation of growth, the earliest sign of E deficiency in the embryo, occurs in the hematopoietic and mesodermal tissues. According to Mason[4] the important physiological action of vitamin E is related to activities of the cell nucleus. The exact role has not been defined, but it is thought that the vitamin is concerned primarily with cell maturation and differentiation. The early conception of the function of vitamin E, the "fertility" vitamin, is based upon the histopathological changes which occur in the testes of vitamin E-deficient rats. There is a typical lysis, the chromatin undergoes definite changes, and the nuclei become crescentric. The effects of vitamin E upon fertility (of rats) have been studied in great detail. The changes appear earlier in the males than in the females.[5a,5b]

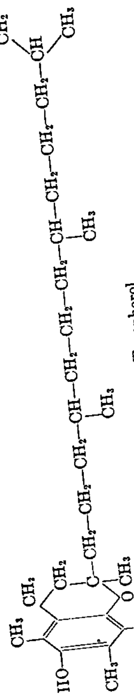
The typical changes in the male are: non-motility of spermatozoa → degeneration with inability to fertilize → degeneration of germinal epithelium → and loss of sex instinct. Once the degenerative changes in the epithelium are histologically evident, they are irreversible and result in a condition known as "functional sterility." No amount of vitamin E leads to arrest or repair of the degenerative process in the male rat.[6a,6b,7] In the female rat, especially in the earlier stages of E-deficiency, there may be a normal oestrus cycle and normal ovulation. Conception and gestation start normally, but the development of the fetuses is usually retarded about the middle of the gestation period, with degeneration of placental and fetal tissues.[5a] This is followed by death and resorption of the young, "resorption sterility." The entire process from estrus to resorption may be repeated and if vitamin E is administered, the animal is able to cast normal litters. If the female animal is continued on an E-deficient diet, there is subsequent degeneration of the uterus[5b] (fibrosis fibromata) and in advanced stages, absolute sterility.

In the early work of Evans and Bishop[2c] it was found that green lettuce leaves, field pea seedlings, whole cereals (oats and wheat), beef liver, egg yolk and wheat embryo contained the new nutritional factor ("X" or vitamin E) and were both prophylactic and curative when fed in reasonable amounts. By extracting the ether soluble material of wheat-germ with 80 per cent alcohol, a brownish oil was obtained which completely cured dietary sterility in doses of about 100 mgms.

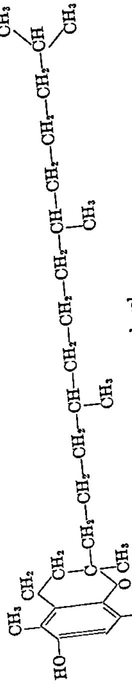
The several vitamin E (tocopherols) are found principally i plant materials; animal tissues contain very little. The riche natural source is wheat-germ oil.[6a] Cottonseed oil and rice ge oil and other seed germ oils contain varying amounts of vitar E;[6a,8,9] the fish liver oils, which are so rich in vitamins A and are very poor sources of tocopherols. Different amounts of several tocopherols occur in the various seed germ oils. cording to Todd et al.[10,11] European wheat germ oil con principally beta-tocopherol while American wheat gerr furnishes mainly alpha-tocopherol. Gamma-tocopherol is principally in cottonseed, palm, and corn oils.[12] Olive o peanut oil contain only traces of the tocopherols.[13]

By means of partition effects between different solvents, a sterol-free concentrate of vitamin E was obtained which was effective in single doses of 10 milligrams. Subsequently, Olcott and Mattill[9] obtained a fraction by high-vacuum distillation which was active in doses of 5 milligrams. Evans et al.[14] and Todd et al.[11] obtained very active preparations by subjecting the crude concentrates to partition between petroleum ether and methyl alcohol. Drummond and co-workers[15,16] prepared active preparations by means of chromatographic absorption, but none of the methods yielded crystalline material. The first solid derivative of the vitamin was obtained when the concentrates were treated with cyanic acid by Evans, Emerson and Emerson.[14] (The esters thus produced are known as allophanates.) By careful purification of the solid thus obtained from wheat-germ oil concentrates, it was found that two compounds (with quite different melting points) were present. These allophanates were also prepared by Drummond and Hoover[15] and by Todd, Bergel and Work.[11] Hydrolysis of the two pure allophanates yields pale yellow oils, both of which are highly active. For these individual vitamin E factors Evans et al.[14] proposed the term "tocopherol"; one of the factors was designated as alpha-tocopherol, the other, beta-tocopherol. A third allophanate was obtained by Emerson and Smith[17] from cottonseed oil which, upon hydrolysis, yields gamma-tocopherol.

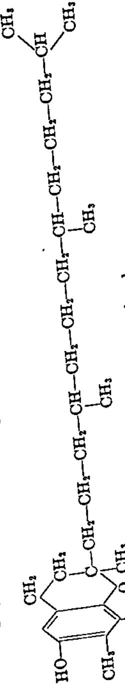
The determination of the chemical structure of alpha-tocopherol by Fernholz[18] in 1938 was followed by the synthesis in the same year by Karrer and collaborators,[19a,19b] Bergel et al.,[20a,20b] and by Smith et al.[21a,21b] The three isomeric tocopherols obtained as oils have not been crystallized. Each readily forms esters, so crystalline compounds of this nature have been prepared in pure form. Each of the tocopherols exhibits a typical absorption spectrum at 295 $m\mu$, the esters show a maximum at 285 $m\mu$. The vitamins E are stable to heat in the absence of oxygen and are not affected by strong mineral acids (H_2SO_4,HCl) at temperatures under 100°C. The compounds are readily oxidized with complete loss of vitamin activity, they withstand regular alkali saponification, are relatively stable to visible light but destroyed by exposure to ultraviolet light.[16] The tocopherols are soluble in



Alpha-Tocopherol



Beta-Tocopherol



Gamma-Tocopherol

all of the usual fat solvents (ether, chloroform, acetone etc.). In addition to the characteristic vitamin activity, the three tocopherols are effective antioxidants. The order of effectiveness as antioxidants is gamma > beta > alpha, just the reverse of their vitamin potency.[22] The synthetic *dl*-alpha-tocopherol can be resolved by esterification and yields a compound identical with the naturally occurring alpha-tocopherol.[19a,19b,23]

The physiological activity of a given vitamin is usually quite specific and any slight changes in molecular structure result in decreased activity or complete loss of the biological properties. In this respect vitamin E is quite different, for E activity is possessed by a large number of widely different natural compounds, only slightly related to the tocopherols in chemical structure. Evans et al.[24a,24b] biologically assayed over 130 compounds (coumarins, phenols, quinones, etc.) and found E activity in over 40 of them. It is still true at the moment of writing that none of the synthetic substances compare with the tocopherols in activity—the effective dose of 3 mgms. for alpha-tocopherol (natural or synthetic) as against 50 and 100 mgms. for several compounds in the above mentioned group.[24,25,26]

The antioxidant action exhibited by the tocopherols is of importance in the commercial preparation and handling of edible fats and oils.[27a,27b] In the early phases of the work on the antioxidant properties it was found that the oxidative changes which accompany the development of rancidity in unsaturated animal fats caused a loss in vitamin A and E content of the fats. It was also noted[28] that certain vegetable oils, especially wheat-germ oil, possessing a number of hydroxyl groups, delayed the oxidation of fats and prevented the development of rancidity and loss of vitamin A. Olcott and Emerson[22] showed that the three tocopherols (alpha, beta, gamma) were effective antioxidants in lard but the antioxidant property of the tocopherols was not proportional to vitamin activity in the rat. A chemical method was used by Hove and Hove[29] to determine the alpha-tocopherol and total tocopherols present in several salad oils (peanut, cottonseed, corn) and in wheat germ oil. In a subsequent paper the same authors[30] reported that at low temperatures (30°) the three tocopherols were approximately equal as antioxidants. At elevated

temperatures (98°) the gamma-tocopherol was several times more active than the alpha-tocopherol. Golumbic and Mattill[31] showed that ascorbic acid (vitamin C) is an effective antioxidant for certain vegetable oils and in spite of its being a water soluble vitamin, it enhances the activity of the tocopherols as antioxidants.

In addition to the effects of alpha-tocopherol (vitamin E) and related compounds upon reproduction in certain animals and the antioxidant action in oils, it has been reported that dietary deficiency of these substances results in muscular dystrophy and pathological changes in the central nervous system of experimental animals (rats,[32,33] rabbits[34,35]). Muscular dystrophy due to a deficiency of vitamin E, has been produced also in young dogs,[36] in guinea pigs,[37] chicks[38] and in ducklings.[37]

Goettsch and Pappenheimer[39] described a neurological condition produced in chicks by a deficiency of alpha-tocopherol (vitamin E). The syndrome, which usually occurs between the 18th and 25th day, is characterized by ataxia, twisting of the neck, clonic spasms of the legs and finally by stuporous death. The condition has been termed "nutritional encephalomalacia." Similar effects have been observed in rats[40,41] maintained on E-deficient diets. Since 1928 muscular paralysis has been associated with a deficiency of vitamin E and histopathologic studies of the muscle tissues of E-deficient animals have revealed lesions affecting principally the striated skeletal muscles. More recently Mason and Emmel,[42] Houchin and Smith[43] have observed myocardial lesions in E-deficient rats. Martin and Faust[44] working with E-deficient rabbits reported an increase in the duration of PR and QT intervals—similar to the effects observed in a young heifer by Gullickson and Calverley.[45] Biopsy experiments[46] (rabbits) indicated that the parenteral administration of alpha-tocopherol (phosphate) reduced the high oxygen consumption of dystrophic muscle by 34 per cent during the first hour, a sharp decline in muscle creatine accompanied the reduced oxygen consumption. The authors suggest that alpha-tocopherol phosphate is intimately associated with unknown enzyme systems which are concerned with the maintenance of the physiological integrity of muscle tissue. Houchin and Smith[43] found that rabbits deficient in vitamin E and showing nutritional muscular dystrophy,

exhibited: (1) a marked increase in sensitivity to posterior pituitary extract; (2) decreased sensitivity to the various cardiac glycosides (digoxin, ouabain); and (3) cardiac dilation. According to Telford et al.,[47] female rats on an E-deficient diet for one year showed a much lower systolic pressure (78 mm.) than normal rats (111 mm.) of the same age.

The *in vitro* experiment of Houchin,[48] with dystrophic hamster and rabbit muscle slices showed that the addition of alpha-tocopherol phosphate to the medium brought about a 40 per cent reduction of the high oxygen consumption. The substance had no effect in the oxygen consumption of normal muscle. In discussing the action of alpha-tocopherol phosphate, Houchin states: "At present any conception of the action of tocopherol must remain highly speculative. In the form of its phosphate it acts as a brake on the oxidative mechanism primarily of skeletal muscle and in its absence these oxidative processes in muscle run riot. An altered creatine metabolism is one of the results of this disturbance." The *in vitro* oxygen consumption by the skeletal muscles of E-deficient rats and chicks was higher than that of muscles from rats and chicks receiving an adequate diet or a protective dose of the vitamin.

The clinical use of vitamin E (tocopherol), by Vogelsang, Shute, and Shute,[49] in the treatment of cardio-vascular disease has been the subject of extensive controversy. The vitamin has been used by this group in the treatment of several hundred cases of heart disease, displaying about every complication and every degree of severity, with improvement in about 80 per cent of the patients. Vitamin E was used with the idea that it decreased capillary permeability and also that it produced local capillary dilation and facilitated tissue oxidation. Probably the outstanding claim was that of marked benefit in coronary occlusion; here, according to this group, improvement was rapid, and further attacks were rare after two weeks of intensive therapy, 200 to 600 mg. or more per day. Clinical studies in the United States have failed to verify the claims made by the Canadian group. Baer et al.,[50] Levy and Boas[51] found no clinical evidence to justify the use of alpha-tocopherol (vitamin E) in the treatment of chronic heart failure or in coronary insufficiency.

Bicknell,[52] Wechsler,[53] Worster-Drought and Shafar,[54] De

Gutierrez-Mahoney,[55] Eaton et al.,[56] Viets et al.,[57] Alpers et al.,[58] Pohl and Baethke,[59] Davison,[60] Meller,[61] and Minot et al.,[62] have used vitamin E concentrates or alpha-tocopherol in the treatment of various muscular dystrophies with varying degrees of success. In the above series studied there were patients with muscular dystrophy, myasthenia gravis, thyrotoxic myopathy, syphilitic amyotrophic lateral sclerosis and the nonatrophic paralysis agitans, none of whom improved appreciably following the administration of wheat-germ oil concentrates or alpha-tocopherol. Steinberg[63] treated 145 cases of primary fibrositis with varying doses of several tocopherol preparations and obtained complete relief of all symptoms in 143 out of the 145 cases. The few (9) receiving intramuscular injections experienced considerable local pain, swelling and soreness. The two patients receiving wheat germ oil were not appreciably improved (ineffective dose, Ed.). The criteria used to establish a diagnosis of primary fibrositis were: (1) muscle stiffness ("jelling") on the least exposure to cold; (2) fibrous nodules or thickening in various planes of aponeuroses; (3) tendon contractures (without known cause) and (4) an increased urinary excretion of creatine which responds favorably to tocopherol therapy. Three early cases of Dupuytren's contracture were markedly improved by tocopherol therapy. Christy[64] made an extended study of the effects of synthetic *dl*-alpha-tocopherol on the usual menopause symptoms and reported that the daily oral administration of 25 to 100 mg. afforded satisfactory relief of symptoms in about 70 per cent of the patients. The vaginal smears were not altered—indicating that alpha-tocopherol is not estrogenic.

Currie[65] used wheat-germ oil extract, in daily doses equivalent to 6 mgms. of alpha-tocopherol, in the treatment of forty cases of "threatened abortion" in women and reported successful births in 90 per cent of the cases treated. Shute[66] reported indefinite results from the use of wheat-germ oil in the treatment of partial, premature separation of placenta—a condition frequently overlooked. There are many causes of abortion in women and it is necessary to take into account too many factors to make it possible to assign to alpha-tocopherol (vitamin E) a specific effect in the prevention of abortion. Shute[66] observed 82 women over periods

of 12 to 18 months and demonstrated, by serologic studies, that about 80 per cent showed a relative deficiency of vitamin E and an excess of estrogenic substances. Shute believes that, in the pregnant woman, vitamin E neutralizes the excess of estrogenic material and thus facilitates intrusion of the placental villi into the uterine wall.

On the other hand, Lubin and Waltman[67] used synthetic alpha-tocopherol in the treatment of 32 cases of threatened, previous, or habitual abortion. In the threatened and previous groups (80 per cent) of the women delivered normal infants.

Vogt-Moller[68] in Denmark, reported that cattle and pigs gave birth to and reared more young when vitamin E was added to the diet. Krauss[69] demonstrated that the lamb-crop was larger and better in a group of ten ewes receiving wheat-germ oil than in the group of ten not receiving the supplement. Willman et al.[70] found that alpha-tocopherol prevented or cured muscular stiffness ("stiff-lamb disease") in lambs. In a group of 17 affected lambs treated with vitamin E there were no deaths, in a group of 8 untreated "stiff lambs," three died and two were so bad they were killed. Wilson, Thomas and Cannon[71] were unable to demonstrate that goats require vitamin E.

Hughes[72] noted a high percentage of nervous impairment and paralysis in the suckling young (rats) born from mothers given barely enough vitamin to complete gestation. The nervous disorders could be prevented by providing generous amounts of vitamin E to the mother throughout the period of lactation. No other vitamin was effective and the extensive experiments established the secretion of vitamin E by the mammary gland. Employing the cure of creatinuria as a "yardstick" Hove and Harris[73] estimated the relative activity of alpha, beta and gamma-tocopherol was in the ratio of 10:3:2. In experimental muscular dystrophy (in rabbits) it required 1.1 mg. of natural tocopherol per kilo per day and 1.4 mg. of the synthetic compound per kilo per day. Mackenzie and McCollum[34] placed the prophylactic requirement of the rabbit at not more than one mg. per kilo per day. Eppstein and Morgulis[74] placed the prophylactic or curative dose for experimental muscular dystrophy (rabbit) at 0.2 to 9.4 mg. per kilo per day.

Quackenbush et al.[75] found that alpha-tocopherol and hydroquinone were equally effective antioxidants for carotene in linolate *in vitro*. However, after water extraction the carotene remained stable only in the tocopherol treated samples. It was thought that the low protection of carotene by hydroquinone in the animal test was due to its rapid extraction in the gastrointestinal tract. It was suggested that many of the discrepancies reported with respect to the response to carotene or vitamin A supplements may be due to a lack of antioxidants, such as tocopherol. Thus olive oil, very low in tocopherol content would be very inferior to cottonseed oil as a vehicle for beta-carotene. Hickman et al.[76a,76b,76c] demonstrated that vitamin E (mixed, natural tocopherols) enhanced the growth-promoting property of vitamin A (alcohol, acetate) and U.S.P. reference oil. The vitamin A activity of beta-carotene was greatly influenced by the vitamin E intake of the experimental rats—0.5 mg. daily of the mixed, natural tocopherols was adequate to demonstrate the sparing action of vitamin E on carotene.

In E-deficient rats Lemley et al.[77] found the average liver storage of vitamin A was 2270 units; feeding alpha-tocopherol to another group resulted in an average liver storage of 3400 units. Increasing the vitamin A intake or extending the period of supplementation diminished the favorable effects of alpha-tocopherol. The work thus agrees with that of Hickman et al.[76] who found that the covitamin activity of the tocopherols is most pronounced with low levels of vitamin A. Growth is also accelerated under these conditions. Mason[78] studied the distribution of vitamin E in three groups of rats: (1) "low-E"; (2) "high-E"; and (3) "excess-E." The intake of vitamin E was estimated as 4,100, and 10,000 times the minimum daily requirement, respectively. The 338 bioassays of various rat tissues showed several interesting facts: (1) on the "low-E" (suboptimal) intake the heart, lung and spleen contained about twice as much E per gram of fresh tissue as the musculature, body fat, and certain organs (kidney, testes, prostate) and about four times as much as the liver. (2) On the moderately high level ("high-E") the E content of all tissues was markedly increased, being especially noticeable in the heart, lung, spleen and greatest in the liver. (3) With excessive amounts of E,

the levels of all tissues were increased. The liver content of E was about 150 times that observed in the "low-E" groups. The experiments also demonstrated that the total storage was only a small fraction of the amount ingested and confirmed the previous suggestion that the tocopherol molecule is either broken down or poorly absorbed.

The most reliable method for determining vitamin E (tocopherol) activity is the biological assay, using synthetic *dl*-alpha-tocopherol as a standard.[6a,8] The chemical method originally proposed by Emmerie and Engel[79] is not very specific and does not differentiate between the alpha, beta and gamma tocopherols, which have very different biological activity. Recent work has made it possible to modify the procedure so that fats,[80] the carotenoids and vitamin A do not interfere and make it possible to determine the alpha-tocopherol activity. Quaife and coworkers[81a,81b,81c] have adapted the chemical method to the determination of tocopherols in blood plasma and milk. Quaife[81c] found that fifteen samples of human milk, collected within one week after parturition, contained 0.13 to 3.6 mg. per 100 cc. of milk or 76 to 1800μg. per gram of fat. These levels fall rapidly. Four composite samples of human milk (from 14 women during first to eighth month of lactation) showed only 37 to 58μg. per gram of fat.

Notwithstanding the considerable amount of work, very little is known concerning the human requirements for vitamin E (tocopherols). The effects of vitamin E on oxygen consumption by various tissues[45,46,48] are very suggestive and may be interpreted as an indication of a definite requirement by man and animals.

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Chapter 4
Vitamins K

It is not uncommon in research work, while working on some planned course, to have a side issue become the main problem. Such an event led to the discovery of an important group of physiologically active substances, or vitamins K. While maintaining hens on highly purified diets, to determine whether fowl could synthesize cholesterol, Dam[1a, 1b] noted that many of the experimental birds developed numerous subcutaneous, intracutaneous hemorrhages and anemia. Horvath[2] observed the coagulation time of chicken blood was materially shortened when the chickens were fed sprouted soy beans. At about the same time McFarlane et al.,[3] engaged in a study of the chicken requirements for the fat-soluble vitamins, observed that the inclusion of ether extracted fish meal in the diet of chicks resulted in heavy mortality and that the blood of the animals receiving the extracted fish meal stood overnight without clotting. When the birds received unextracted fish meal there were no deaths nor were there any changes in the clotting time of the blood. Later, Dam and co-workers[4a, 4b] found that although the condition had considerable resemblance to scurvy, it was not improved by lemon juice (vitamin C plus P) nor was it helped by cod liver oil (vitamins A and D). Fats extracted from rice, corn, rye and sunflower seeds were also ineffective as curative agents whereas the fats extracted from pork liver and hemp seed were very active curative agents. The active substance was designated "vitamin K."

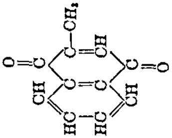
Working independently, Almquist and Stokstad[5] made similar observations and showed that the active material was present in the tissues of several plants; green grass, spinach, cabbage and alfalfa. Subsequently, Almquist[6a, 6b, 6c] prepared a very active, purified extract from alfalfa which became the first preparation available for clinical trail. The experimental data of Dam et al.

Almquist and co-workers strongly suggested that the hemor syndrome produced in chicks was a close counterpart o hemorrhagic conditions observed in man. Dam et al. monstrated that vitamin K plays an important role in re and maintaining normal levels of blood prothrombin in ex ntal animals (chicks) and within a short time several group cal investigators established the use of the new vitamin ir atment of certain hemorrhagic conditions in man. Witl nd more of the organic chemists showing an interest in the 1 field, it was not surprising to have the pure substance iso :dentified and synthesized in a very short time. It soon evident that there were at least two vitamins K. Dam solated pure vitamin K_1. (2-methyl-3-phytyl-1,4-naphtho e) and the Doisy group[9] isolated pure vitamin K_2 (2-methyl- 1esyl-1,4-naphthoquinone) and established its structure.[10] nthesis of K_1 was accomplished almost simultaneously by ifferent and independent groups[11,12,13] and about the same nsbacher and Fernholz[14] discovered that 2-methyl-1,4- oquinone possessed marked antihemorrhagic activity.

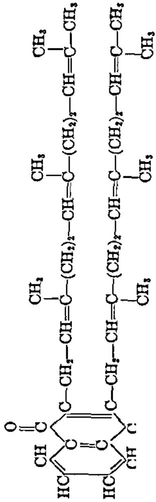
41 the Council on Pharmacy and Chemistry[15] selected and 1 the term "Menadione" as a nonproprietary name for the yl-1,4-naphthoquinone compound. Menadione (K_3) and one sodium bisulfite are "official" in U.S.P., XIV (pages332, Menadione is used for oral therapy and the water-soluble one sodium bisulfite is employed in parenteral therapy. nsbacher and Fernholz[14] found that 2-methyl-1,4-naph- one possessed exceptional K activity and since it could be prepared in a pure state, the Doisy group[16] and Almquist se[17] adopted the compound as a standard of reference in g K activity of other substances. Since 2-methyl-1,4- quinone, "menadione," is extensively used clinically, ention of the physical and chemical characteristics is in-

The material used commercially is a light yellow crys- powder with a melting point of 106°C. It possesses a eristic odor and is sparing soluble in water (about 13 mg. cc.). Vitamin K_1 and K_2 are soluble in the common fat (ether, petroleum ether, acetone) but are insoluble in nd only slightly soluble in methyl alcohol. They are fairly

K_2 occurs as yellow crystals which melt a
K_1 and K_2 exhibit typical ultraviolet al
maxima between 243 and 328 mμ.

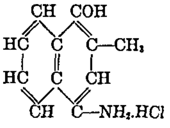


MENADIONE
VITAMIN K_3
2-Methyl-1,4-



Vitamin K_1

Vitamin K_2



VITAMIN K_5
2-Methyl-1-oxy-4-amino-naphthalene hydrochloride

It soon became evident that the highly specific physiological action was not restricted to the two compounds, vitamin K_1 and vitamin K_2. Extensive chemical studies indicated that various quinones could be converted into corresponding hydroquinones or esters without pronounced loss of effectiveness, provided that the compounds could be readily reconverted to quinones. Substitution of the benzene ring or replacement of the 2-methyl group by higher alkyl groups caused marked loss of activity.

On the basis of the amount of material required to reduce the blood clotting time of K-deficient chicks to less than 10 minutes during an 18-hour period (in 60 to 80 per cent of the experimental animals), Fieser and co-workers[21,22a,22b] compared the K activity of several different types of substituted compounds, some prepared by themselves and other compounds coming from the laboratories of Ansbacher,[14] Doisy,[23] Almquist[24a,24b] and Karrer.[25] So much space would be necessary to fully cover the references dealing with the 100 or more compounds that it is necessary to limit the number presented. The activity of a few of the different substituted compounds is shown below:

Compound	Effective dose in μg.
2-Methyl-3-phytyl-1,4-naphthoquinone, vitamin K_1	1.0
2-Methyl-3-difarnesyl-1,4-naphthoquinone, vitamin K_2	1.6
2-Methyl-3-farnesyl-1,4-naphthoquinone	5.0
2-Methyl-3-cinnamyl-1,4-naphthoquinone	25.0
2-Methyl-3-benzyl-1,4-naphthoquinone	200.0
2-Methyl-1,4-naphthoquinone	0.3
2-Methyl-1,4-naphthohydroquinone	0.5
2-Farnesyl-1,4-naphthoquinone	500.0
2-Ethyl-3-phytyl-1,4-naphthoquinone	1000.0
Vitamin K_1 oxide	1.2
5,8-Dihydro-vitamin K_1	4.0

Anyone interested in exploring this work in detail can easily start from the references included.

Emmett et al.,[26] Sah and Brüll,[27] synthesized 4-amino-2-methyl-1-naphthol (K_5). It was found by chick tests that 4-amino-2-methyl-1-naphthol was three to four times as potent as vitamin K_1. It was also very effective when used intravenously in the treatment of obstructive jaundice. Weir et al.[28] used vitamin K_5 intravenously in doses of 1–2 mg. in 45 patients (including 26 cases of jaundice, six cases of cirrhosis and 13 cases of gastro-intestinal and miscellaneous lesions) with excellent results and pointed out that any failures to adequate K therapy are indicative of irreversible hepatic injury. Because of low toxicity and marked fungistatic properties Pratt[29] et al. suggested that vitamin K_5 (4-amino-2-methyl-1-naphthol hydrochloride) might be valuable for the clinical treatment of dermatophytic fungi and that it might well be used for the preservation of foods and beverages. In a subsequent paper[30] it is shown that vitamin K_5 is a strong bacteriostatic agent since from 10 to 300 parts per million inhibited a number of the common bacterial and fungal despoilers of food products. Pure K_5 crystallizes out of hydrochloric acid as colorless needles which after a few hours acquire a light pink color (melts at 280°C. with decomposition). Vitamin K_5 is readily soluble in cold water. Aqueous solutions are unstable in the presence of light and air but may be heat sterilized.

The vitamins K, or compounds possessing K activity are necessary to maintain a normal blood clotting time. The coagulation of blood is not a simple, single reaction but involves several substances and at least two enzymes. According to Dam et al.[4b,7] prothrombin contains no vitamin K nor does the vitamin cause *in vitro* coagulation of blood. Since vitamin K deficiency produces a lowering of the prothrombin concentration, it is assumed that K is involved in the formation of prothrombin. It also seems quite certain that the synthesis of prothrombin is a function of the liver[31] for it has been observed that partial extirpation of the liver[32,33] results in a marked decrease in prothrombin concentration. Quick[34] has shown that an excessive ingestion of vitamins K does not increase the blood prothrombin above a certain level, indicating a special mechanism for the formation of prothrombin.

Vitamin K deficiency in man and animals is characterized by (1) a decrease in the prothrombin content of the blood; (2) marked prolongation of the clotting time and (3) by the occurrence of hemorrhages. In chicks the hemorrhages are observed in all parts of the body.[34] In man the hemorrhagic syndrome due to K deficiency is frequently observed in the newborn infant and persists until a normal intestinal flora is established and able to supply the body needs.[36,37a,37b] Low prothrombin levels in man are observed under conditions which impair absorption[38a,38b] (sprue, celiac disease), whenever there is liver damage[32,33] (cirrhosis, hepatitis, carcinoma) and in obstructive jaundice[39,40] and biliary fistula.[41]

While a blood clot may seem so commonplace that no discussion is needed, it may be well to point out that the chemical and physical changes involved in the coagulation of blood have been the subject of much speculation and a considerable amount of investigation. It is now generally believed that coagulation takes place in two distinct but consecutive reactions. According to Eagle[42] the steps are: (1) prothrombin (a thermolabile plasma protein) + calcium ion + thromboplastin (widely distributed as a intracellular substance and abundant in blood platelets) = thron bin; and (2) fibrinogen (a labile plasma protein) + thrombin fibrin. The coagulation of blood is influenced by a number factors which act to increase the formation of fibrinogen, p thrombin or the platelet factors (thromboplastin). The admi tration of vitamins K, or compounds possessing K activity (m dione), increases the prothrombin content of the blood. A concentration of prothrombin may result from: (1) a deficien vitamin K (the hemorrhagic chick syndrome[14,19]); (2) i "sweet clover disease" in cattle[43] and (3) in association liver damage (obstructive jaundice, biliary fistula).

Two biological procedures constitute the principal mea determining vitamin K deficiency or activity. (1) Estima spontaneous clotting time; while the clotting time of norm blood is about four to six minutes, the blood of K-deficien may not clot for several hours.[35] The K-activity may mated by either the prophylactic dose or the curativ (2) Determination of the prothrombin content of the b

This method has the advantage of being free from numerous variables which may affect spontaneous clotting time.

Several clinical procedures[40,44,45] are available for measuring blood coagulation: (1) "Coagulation Time." This method[46] determines the clotting of blood *per se*, without any tissue factors and is subject to a number of modifying factors; (2) The Howell test[47] for "prothrombin time." This procedure measures the coagulability of the blood plasma and is not specific for prothrombin. Nygaard[48] has modified the original Howell method and has also devised an instrument for measuring the endpoint (coagulation); (3) The quantitative estimation of prothrombin by the two-stage procedures developed by Warner, Brinkhous and Smith.[49] For clinical practice this method is too time-consuming and one finds the Quick procedure[34,44,45] in general use. In brief the method[50] is as follows: four and a half cc. of blood, withdrawn rapidly and with special precaution to avoid trauma, are promptly and thoroughly mixed with 0.5 cc. M/10 sodium oxalate and centrifuged at low speed for five minutes. Of this plasma 0.1 cc. is transferred to a dry, clean test-tube (13 x 100 mm.) mixed gently with 0.1 cc. of thromboplastin solution and the mixture is warmed in a water bath kept at 37.5°C. Then 0.1 cc. of M/40 calcium chloride is added, and the tube quickly shaken. A stop watch or clock is started at the instant the calcium chloride is added, and the exact time required for the formation of a firm, semi-solid clot is recorded. In order to determine the seconds at which the clot is developed, the tube should be tipped every few seconds. Quick uses the following formula for calculating the prothrombin concentration, expressed as per cent of normal:

$$\text{prothrombin concentration} = \frac{302}{\text{clotting time} - 8.7}$$

Aggeler et al.[51] pointed out that the lack of experience with the test and inadequate standardization of the thromboplastin constitute the principal sources of error in all methods of determining the prothrombin concentration. For reliable results a normal standard should be based on not less than five individual samples of blood and all tested with the same sample of thromboplastin.

Vitamins K_1 and K_2 are fat-soluble and thus are best absorbed

under the conditions which favor the absorption of fats; normal functioning of the liver and the presence of bile salts are important factors in the absorption of the *vitamin* K.[52,53a,53b,54] The fat-soluble K compounds administered orally are not absorbed by rats with bile fistula unless bile salts are given simultaneously. Elliott et al.[55] showed that the administration of large amounts of mineral oil with vitamins K interferes with normal absorption and utilization. In this respect it is necessary to distinguish the fat-soluble vitamin K from the water-soluble compounds possessing K activity. The latter are absorbed readily from the gastrointestinal tract and do not require the presence of bile salts.[56,57] The results of extensive experiments indicate that in the absence of an adequate diet severe K-deficiency states many occur in animals when *bacterial activity is reduced* in the intestinal tract. The reduced bacterial activity may be produced by sulfa drugs or by surgical removal of the ceca or rumina.[58]

The natural vitamins, K_1 and K_2, are non-toxic even in very large doses[59] (mouse, 25 Gms. per kilo) but the much used 2-methyl-1, 4-naphthoquinone (menadione) and the 2-methyl-1,4-naphthohydroquinone produce definite toxic symptoms when given to man, dogs, rabbits or mice in excessive amounts.[59]

Plants and many microorganisms produce varying amounts of vitamin K_1. In plants the synthesis is apparently associated with chlorophyll and photochemical reactions occurring in the green leaves; carrot tops are rich in vitamin K_1 but the roots contain only traces, the other leaves of cabbage contain about 14 times as much K_1 as is found in the inner leaves.[60]

Man and animals acquire the vitamin K through the food in*take and through bacterial* synthesis *in the intestinal tract*. Man and animals do not have any special storage organs and the vitamins K are apparently metabolized in considerable amounts. Neither K_1 nor K_2 is excreted via the urine,[8] the feces contain considerable amounts, varying with the extent of bacterial synthesis. Vitamins K are secreted in the milk of mammals and appear in the eggs of fowl.[61] The vitamins are apparently not required by most microorganisms but are required by man[62] and all animals thus far studied (dog, rabbit, rat, mouse, pigeon, chick, turkey). Under normal conditions the bacteria present in the intestinal

tract provide an abundance of vitamin K-active substances and except in pathological conditions man needs no external supplies of vitamin K.

Good food sources of vitamin K are spinach, kale, cabbage and cauliflower. Pork liver is the richest known animal source[63] of vitamin K whereas chick and rat livers[62,63] contain very small amounts of K.

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Chapter 5

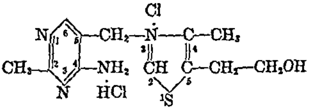
Thiamine Hydrochloride (Vitamin B_1)

All historical accounts of the several vitamins include reference to the early (1885–87) experimental work of Takaki, in which he was able, by changing the diet, to cure and prevent beriberi in the Japanese Navy. Eijkman[1] is credited with the earliest (1897) production, cure and prevention of a disease by dietary changes. In 1912 Funk[2] produced, by dietary means, polyneuritis in pigeons and was able to cure the symptoms by administering aqueous extracts of rice bran. During the early years, this member of the B complex was referred to under various names: the antineuritic vitamin; the anti-beriberi vitamin; polyneuramin; and oryzanin.

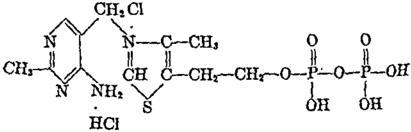
Crystalline vitamin B_1 was first isolated from rice bran by Jansen and Donath[3] in 1927, subsequently Windaus and co-workers[4] isolated crystalline vitamin B_1 from yeast and indicated the empirical formula. However, elementary composition remained uncertain until Williams established the structure[5] and later, with his coworkers, reported the synthesis of vitamin B_1.[6] The problem of a suitable name for the pure crystalline vitamin, first isolated by Jansen and Donath,[3] created much discussion and some confusion. In 1935 Jansen[7] wrote: "Further, I propose to call the present vitamin B_1 in the future aneurin (from A [nti-poly] neur [itis vitam]in). Think I have some right to propose this as Dr. Donath and I were first to obtain this vitamin in crystalline form." The Council on Pharmacy and Chemistry[8] objected to the term *Aneurin* but subsequent correspondence between the Council and Dr. Jansen finally resulted in the selection of the term *Thiamine Chloride.*

Thiamine hydrochloride, vitamin B_1, is very soluble in water (one gram per ml) and glycerin (one gram per 18 mls) but is insoluble in acetone, ether or chloroform.[3] From hydro-alcoholic

solutions the pure vitamin crystallizes as needles (the hemihy drate) which melt at 248–250°.[6] When exposed to ordinary con ditions of temperature and humidity, the crystals absorb about mole of water. The crystalline vitamin and all solutions con taining the vitamin possess a characteristic yeast-like odor. Th vitamin is relatively stable in acid solutions, at pH 3.5 thiamin solutions may be heated at 120°C. without decomposition.[9] I less acid solutions there is definite destruction of the vitamin an in neutral or alkaline solutions it is rapidly destroyed by hea The vitamin is very susceptible to both oxidation and reductio The druggist should also note that tannic acid precipitates thia mine and since wine contains variable amounts of tannic aci they should not be used as the menstruum in preparations con taining thiamine.



Thiamine (B_1)



Cocarboxylase (Vitamin B_1 pyrophosphate)

The physiological effects appear to be related to the specific mo lecular structure for alterations such as those caused by oxidation to the yellow fluorescent thiochrome[10] or the two cleavage products produced by sodium sulfite and the reduced dehydrovitamin result in inactive products. The principal biological activity of the vitamin is associated with the readily formed pyrophosphoric acid ester, the coenzyme, cocarboxylase.[11] The complete carboxylase system consists of a specific protein (apoenzyme), the carboxylase

(coenzyme) and certain metals. Manganese, magnesium and iron act as stimulants whereas calcium, zinc, nickel, and cobalt retard (in low concentrations) or accelerate (in moderate concentrations) the reaction, depending on the amount present.[12a,12b] Excessive amounts of the various metal ions completely stop the reaction (irreversible). In this connection Eggleton[13] found an abnormally low zinc content of tissues (cells) during periods of B_1 deficiency. Green and coworkers[14a,14b] isolated highly purified cocarboxylase from top brewer's yeast, a diphosphothiamine-magnesium-protein.

So far as is known, and except for sheep and cattle (ruminants), all animals need a dietary supply of thiamine. The vitamin occurs in the animal body in the free form and as esters. Thiamine and the pyrophosphate ester (cocarboxylase) also occur in combination with protein.[15] Small amounts (one *μg.* per 100 mls.) of free thiamine are found in the blood plasma and in spinal fluid.[16] The blood cells contain cocarboxylase, the blood plasma contains only the free vitamin. It is probable that all nucleated cells have the ability to phosphorylate thiamine;[15] liver, kidney, brain and muscle tissues are able to form cocarboxylase.[17,18]

Although many synthetic compounds, differing only slightly in structure from thiamine, have been prepared,[19] not one has possessed the characteristic and complete vitamin activity.[20] It is apparent that the pyrimidine and thiazole rings with the methylene bridge between,[19] the unsubstituted amino group (4-position in pyrimidine ring), the 5-hydroxy-alkyl-group and the free 2-position in the thiazole ring[19,21] are essential for complete vitamin action.

All higher plants have the ability to synthesize thiamine. According to Pyke,[22] plant leaves, regardless of botanical family, contain a fairly constant value of about 75 *μg.* of thiamine per 100 grams of leaf. The greatest concentration of thiamine is found in the outer integuments of the seeds. Roots are unable to synthesize the vitamin but require thiamine for continued cell division.[23] Many plants not only synthesize the vitamin but excrete considerable quantities through the root[24] system and as a result, the soil adjacent to these plants supports a much higher concentration of microorganisms than is observed outside of the area af-

fected by the plant secretions.[24] Thiamine may be regarded as a true plant hormone.

The pyrophosphoric ester of thiamine is specifically involved in the utilization of pyruvic acid, an intermediate degradation product of carbohydrate metabolism (see review by Peters[25]). In many plants, some bacteria, and in all animal tissues the first reaction is the carboxylation of pyruvic acid.[26] A series of enzymatic reactions carry through the "citric acid cycle"[27] or through a variant, the "succinic acid cycle."[28] The final reactions result in the total oxidation of the pyruvic acid to carbon dioxide and water. In animal tissues the reactions of both the citric acid and the succinic acid cycles are in progress at the same time but at very different rates. In quantitative experiments, utilizing brain tissue, Long[29] found that 67 per cent of pyruvic acid was completely metabolized to carbon dioxide, according to the citric acid cycle and 29.5 per cent passed through succinic acid cycle, yielding carbon dioxide, lactic and acetic acids.

Although there are relatively high concentrations of thiamine in animal livers, the kidneys, heart muscles and brain,[30a,30b] the animal body lacks any important storehouse and the total amount present provides for only a few days of normal function, thus a daily intake is essential. On an abundant diet not all of the thiamine ingested is absorbed; after the body needs are met the remainder is excreted via the urine (one cannot exclude some destruction). On an average diet a normal person excretes *60 to 240 μg. daily.* Thiamine deficiency results in a greatly decreased urinary excretion. Vitamin B_1 pyrophosphate(cocarboxylase) is not normally excreted in the urine.

Wherever nutrition is on a low level one may expect to find various vitamin deficiencies, including the signs and symptoms caused by a lack of thiamine. In the Orient, where polished rice continues to be the staple article of the diet, one still sees severe cases of thiamine deficiency. In the United States the only severe cases are usually found in alcohol addicts. Up to recent times it was generally believed that there were no frank thiamine deficiencies in the Western World; the only admitted instances were the rare cases of classical beriberi and even in those cases it was necessary that there be no other etiological factor than a grossly

inadequate diet.[31a,31b] Similar clinical syndromes caused by impaired absorption from the gut, necessary restricted food intake, excessive excretion (diarrhea), were designated as polyneuropathies and were further qualified by affixing a label to describe the supposed etiology—thus "infections polyneuritis," "alcoholic polyneuritis," "polyneuritis of pregnancy," etc., were commonly used terms.[31b] The recognition of these polyneuropathies as a part of the beriberi syndrome brought out, for the first time, the incidence of nutritional failure in this country. Jolliffe and Goodhart[31b] point out that the usual type of neurologic examination (papillary reflexes, plantar response, knee jerks, etc.) most invariably misses the milder manifestations of nervous system changes (bilateral tenderness of the calf muscles, absence of ankle jerks, plantar dysesthesia). As far as the clinical picture of thiamine deficiency is concerned, the earliest objective signs are plantar dysesthesia and tenderness of the calf muscles. The plantar dysesthesia is an unmistakable hyperesthetic pain and not the common tickling sensation elicited by scratching. In the presence of even mild thiamine deficiency the calf muscle becomes very sensitive to pressure and vibratory sensation in the toes is diminished. These signs are most suggestive and when, in addition, ankle jerks are absent, a very definite diagnosis of thiamine deficiency can be made. Extension of the deficiency results in the loss of knee jerks; the loss of vibratory sense may extend as high as the pelvis and usually there is evidence of impairment of position sense. Long-continued thiamine deficiency results in a progression of the neurologic involvements: the upper extremities are affected, calf muscle atrophy is marked; toe and foot drop are usually evident; *and walking is difficult or impossible.*[32,33]

Summing up the nervous system changes caused by thiamine deficiency, the lesions, whether mild or severe, are bilateral and symmetrical, involving first and predominantly the lower extremities. Jolliffe[33] would exclude from the thiamine-deficient group: peripheral neuritis involving a single nerve, neuritis not bilateral and symmetrical, and neuritis not observed first in the lower extremities.

Beriberi has been defined by Jolliffe and Rosenblum[34] as "a clinical syndrome which follows failure to ingest, absorb, or utilize

sufficient amounts of vitamin B_1. It is, as a rule, associated with prolonged and excessive use of a diet rich in calories derived from refined carbohydrates or alcohol. It is characterized clinically by varying degrees of a peripheral, symmetrical polyneuritis which may occur alone or in combination with edema, serous effusions, enlarged heart and circulatory failure." This definition suggests the various clinical types of disease: (a) "dry" beriberi, in which the signs are restricted to the nervous system; (b) "wet" beriberi, in which the polyneuritis (mild to severe) is accompanied by edema, and serous effusions in the pleural, pericardial or peritoneal spaces; (c) "cardiac" beriberi, in which polyneuritis is associated with heart failure; and (d) "mixed" beriberi, in which one may find any one or various combinations of the above.

In clinical practice, edema without signs of congestive failure is the more commonly observed type of cardiac disturbance resulting from thiamine deficiency. The edema is usually limited to the ankles, and since there are no signs of either heart or renal disease, the condition is perplexing to many physicians. This mild (pitting) edema, as well as the more extensive type, usually responds favorably to bed rest. Cardiac beriberi usually manifests signs of both right and left heart failure (signs of right heart failure usually predominate), precordial pain, edema and serous effusions, dilated cervical veins and frequently pulmonary congestion. Circulatory collapse and sudden death may occur without warning, especially in ambulatory patients.[34,35]

The Wernicke syndrome, part of which is now recognized as due to thiamine deficiency,[36,37] is characterized by varying degrees of ophthalmoplegia, ataxia, polyneuropathy and clouding of consciousness. Although the early reports did not list alcohol as the main causative agent, it is now generally agreed that it is the principal etiological factor. Other factors include severe gastrointestinal disturbances, such as vomiting and diarrhea. The pathological lesions of Wernicke's disease are confined to the periventricular gray matter and consist in small foci of degeneration and varicose changes of the blood vessels.[36,37] Alexander[38] has demonstrated that the pathological changes produced in pigeons by a thiamine-deficient diet are identical with those observed in the Wernicke syndrome in man. Jolliffe et al.[36,37] came to the

conclusion that the Wernicke syndrome is essentially a nutritional deficiency disease and that it is frequently a combination of several deficiencies: pellagra, scurvy, nicotinic acid encephalopathy and ariboflavinosis. Naturally, each of the cardinal symptoms requires specific treatment; the ophthalmoplegia, the polyneuropathies and usually the clouding of consciousness respond favorably to thiamine therapy. Frequently the entire B complex is necessary for the alleviation of all of the signs and symptoms.

Clinical experience of the last few years indicates that thiamine deficiencies, with no demonstrable anatomic basis, cause a greater number of disturbances of function than had been supposed. The symptoms are commonly found in the group of patients which have been classified by the physicians as "having nothing organically wrong" and have been put in the "grab-bag" of "neurasthenics" and advised to "forget their ills." As is well recognized, the syndrome is principally that of tension, irritation and general weakness; the patient complains of insomnia, fatigue, irritability and various aches and pains; there are also many complaints of constipation, loss of appetite, change in the texture of the skin and irregularities of the heart. Jolliffe et al.[39] were able to produce a neurasthenic syndrome in four out of five experimental subjects (internes). Symptoms and signs (appearing on the 4th and 5th days) included fatigue, lassitude, precordial pain, dyspnea on exertion, skin dysesthesias and calf muscle tenderness. The addition of thiamine to the experimental diet caused all of the symptoms and objective signs to disappear within six days. Williams, Mason and Smith[40] maintained four subjects (women) on a B_1-deficient diet for 21 weeks and produced definite symptoms and signs (fatigue, loss of weight, anorexia, constipation and calf muscle tenderness) in each of the four subjects. All of these conditions were promptly alleviated by the subcutaneous administration of thiamine, four daily doses of one milligram each. A study of urinary excretion during the experimental period and during the period of thiamine therapy indicated that when the tissues were saturated, absorption of the vitamin by the tissues was minimal and excretion was greatly increased. Later Williams et al.[41] repeated the studies with six subjects (women) representing as far as possible the various types of

individuals, the study extending over a period of 88 days. It was noted that the more active (mentally and physically) persons *were the first to experience* symptoms of deficiency. The syndrome was characterized by: mental depression, general weakness, dizziness, precordial pain, dyspnea, anorexia, nausea, loss of weight, insomnia, calf muscle tenderness and roughness on the skin. The diet was complete in all other respects; there was no evidence of deficiencies of riboflavin (cheilosis) or nicotinic acid (reddening of tongue). Subjective improvement was observed in every case a few hours after one mg. of thiamine was given subcutaneously and *at some time during the 18 days of thiamine* therapy all of the symptoms and signs, developed on the restricted diet, disappeared. The authors concluded that the disease brought about by a deficiency of thiamine resembled in minute detail the disorder which "the discriminating psychiatrist designates as neurasthenia." The authors also mentioned that since classical beriberi of the oriental type is not commonly observed in this country, thiamine deficiencies will be found often where a *diagnosis of "neurasthenia" or "anorexia nervosa"* has been *made. It is not desired to convey the impression that all so*-called "neurasthenia" is due to thiamine deficiency—such is definitely not the case.

As several substances are involved, it is rather difficult to determine the exact role played by each of the components of the "B complex" in the nutritional support of growth, reproduction and lactation. The most important physiological functions of thiamine are: the prevention and cure of polyneuritis;[1,2] the promotion of growth; a marked effect upon appetite (thiamine deficiency); functional maintenance of normal movements of the digestive tract (this includes the contractions of the stomach and motility of the entire intestinal tract); and since phosphorylated thiamine (cocarboxylase) is necessary in the metabolism of pyruvic acid[25] (intermediary metabolism of carbohydrates), thiamine is of great importance to the functioning of cellular activity in all tissues and systems within the animal body.

Thiamine deficiency symptoms in rats resemble many of those observed in man. In addition the young show marked retardation of growth. Pigeons (used very early for vitamin B_1 assays)

take of thiamine was 40 per cent (0.5 mg.) of the recommended allowance. Two of the 15 subjects studied showed plantar dysesthesia, possibly the earliest objective evidence of thiamine deficiency. The authors noted that in such groups the mother is usually the worst fed member of the family. Lane, Johnson and Williams[48] studied the thiamine content of 57 foods commonly used by the average American family, also 15 different diets selected from these 57 foods, and estimated that the average diet of 65 to 70 per cent of the population, contained 0.8 mg. of thiamine per 2500 calories. This figure, considerably lower than generally believed, can easily be increased to 1.3 mg. per 2500 calories by the general use of vitamin enriched flour and/or bread.

In any nutritional survey it is important to keep in mind the losses of the various vitamins which take place during the preparation, cooking and, most of all, the deleterious effects of long-time holding on steam tables. Heller, McCay and Lyon,[49] using a Brooklyn Navy Yard cafeteria feeding industrial workers, made a rough estimate of the effects of the mass handling of foods. The studies covered the purchase of raw and frozen vegetables (frozen peas, lima beans, green beans, spinach, raw carrots, potatoes and cabbage) during November and December of 1942. Samples were taken as follows: (1) frozen foods before defrosting or sample of raw product; (2) immediately after food was cooked; (3) cooking water, right after cooking; and (4) sample from steam table when serving period ended. The survey showed careless holding of vegetables (potatoes peeled the day before), excessive use of water in defrosting (water discarded) and prolonged holding (as long as 165 minutes after cooking completed) on the steam table. The loss of thiamine, in the foods studied, varied from 16 to 64 per cent. Sarett et al.[50] studied foods served in three restaurants, representing three contrasting price groups and found no significant differences in the vitamin content of the foods served. Fresh fruits or fruit juices were required to meet the vitamin C requirements and only special selection of foods provided an adequate intake of thiamine.

McIntire et al.[51] studied vitamin retention of meats cooked under standard conditions and found an average of 74 per cent of thiamine retained during the roasting or broiling of pork loins

and hams. These authors noted the difficulty of comparing the vitamin content (mg. per gram) of fresh meat because of wide variation exhibited by different pork carcasses and also in the fat content of the particular portions used in a study. Schweigert and associates[52] studied the thiamine content of hams during storage, curing and cooking. Under commercial storage conditions there is a loss of 10 to 15 per cent of thiamine (also same amount of riboflavin and niacin); the several steps in curing caused the loss of 17 per cent of the thiamine and, when roasted, the loss of thiamine was 42 per cent. The same authors[53] studied vitamin retention during the cooking of lamb and veal and found that roasting or broiling caused a loss of 57 to 70 per cent of the thiamine. Rice and Robinson[54] found that dehydrated pork retained 63 per cent of the thiamine and dehydrated beef retained 76 per cent of the original thiamine content. Canned pork meat, stored for 10 months at 80°F. retained only 52 per cent of the original thiamine value. Feaster et al.[55] found that the various heat processes caused as much as 48 per cent loss of thiamine and that thiamine retention of heat processed pork during storage was influenced by temperature and time; storage for one year at 98°F. resulted in 80 to 88 per cent loss of the thiamine.

Holmes and associates[56] observed no significant loss of thiamine in milk (3 μg. per 100 cc.) during pasteurization (32 samples); Knott[57] reported 2.6 μg. of thiamin per 100 cc. in pasteurized cow's milk, 1.8 μg. per 100 cc. of evaporated milk, an average of 20 μg. per 100 cc. of properly collected breast milk.

Since frozen foods are fast becoming important items in every household it is well to investigate the effects of the freezing process and subsequent storage upon the vitamin content. Fellers et al.[58] found that the quick freezing of peas and spinach resulted in little or no loss of thiamine (2 to 6 per cent) but reported a large loss of thiamine (25 to 50 per cent) during the quick freezing of lima beans and asparagus. However, Harris et al.,[59] working with various methods for processing foods, were unable to detect any thiamine losses in vegetables quick frozen by commercial processes. Likewise, Lee, Gortner and Whitcombe[60] found no significant changes in the vitamin content of peas and snap beans during quick or slow freezing nor after storage of same at 60°F. for six months.

The frequent reliance of the average household on canned foods makes it advisable to briefly survey the effects of canning processes on as many as possible of the commonly used items. Guerrant et al.[61] co-operated with the canneries of New York, Delaware and Maryland during the 1943 and 1944 canning seasons. The study included asparagus, green beans, wax beans, lima beans, peas, carrots, whole kernel corn, tomatoes, tomato juice and sour cherries. Except for tomatoes, large losses of thiamine occurred during the canning processes: asparagus, 50 per cent; lima beans, 80 per cent; corn, 80 per cent; peas, 65 per cent; but tomatoes only 12 per cent. These losses reflect the instability of the vitamin in essentially neutral media and the favorable influence of an acid medium. Fellers et al.,[58] Harris et al.,[59] and Lamb et al.[62] also found that canning processes resulted in heavy losses of thiamine—as much as 70 per cent loss in lima beans. General cooking methods for vegetables used in the home also result in heavy losses of thiamine, a considerable part of the total loss of B_1 occurs when the cooking water is discarded. Table 1, page 85 data of Aughey and Daniel,[63] illustrates losses of thiamine by different cooking procedures.

Due to the various factors which influence B_1 requirements, Williams et al.[64] feel that long-term studies are necessary and that requirements should be based on: (1) physical and psychologic status; (2) condition of stores in the tissues; (3) biochemical status of subjects who have been maintained several months on known intakes of B_1; (4) and long-term maintenance on measured diets and under uniform environment. The determination of excretion of B_1, after subcutaneous administration of a test dose of 1 mgm., was considered a satisfactory method for ascertaining condition of the tissue stores of B_1 and cocarboxylase. Estimation of blood pyruvic acid after administration of dextrose (intravenously or orally) was considered a valid test for the detection of a biochemical defect due to B_1 deficiency (excluding other causes of abnormal elevation of pyruvic acid). Experiments with five subjects maintained on a diet of carbohydrate, fat, and protein, conventional for majority of American diets, showed that an intake of B_1 of 0.45 mg. per 1,000 calories cannot be regarded as more than the minimum daily requirement. In order to provide

for higher proportion of non-fat calories, individual variations, and various causes of increased requirements, these investigators regard the daily allowance of 0.6 mg. per 1,000 calories (recommended by the Food and Nutrition Board of the National Research Council) as none too much. See Table II, page 242.

Holt and co-workers[65] point out that the variations in vitamin requirements exhibited by normal subjects, the effects of pathological processes on absorption and utilization, and the increased need for certain factors have complicated the problem of establishing human requirements. The artificially fed infant, receiving a relatively constant diet, obviates many of the factors mentioned above. Using the 24 hour excretion of thiamine as a testing procedure, it was found that the artificially fed infant requires 0.14 to 0.20 mg. of thiamine per day. According to Friedemann et al.[66] there appear to be very definite limitations to human intestinal absorption of thiamine. In a study of 14 human subjects, the maximum which could be taken orally without an increase of fecal thiamine was about 5 mg. daily, less than 2 mg. (including thiamine in food) at each meal. With a total intake of 5.1 to 5.2 mg. per day, the urinary excretion was greater when the vitamin was given with meals than when given one hour previous. With an intake of 19 to 40 mg. per day (in divided doses) 8 to 14 mg. (mean of 11.2 mg.) were absorbed or not recovered in the feces. The maximum economical intake-level seemed to be about 5 mg. per day. In normal subjects receiving from 7 to 40 mg. supplement daily, the maximum quantity destroyed was about 4 mg. per day. The principal site of thiamine destruction is in the tissues; some may be destroyed in the colon. Thiamine mononitrate behaved like thiamine hydrochloride.

Harris and Leong[67] studied the urinary excretion of thiamine in normal adults and found that the amount excreted via the urine was approximately 5 to 8 per cent of the amount ingested in the food. They were of the opinion that the urinary excretion of 36 micrograms or less of thiamine per day was an indication that the diet was deficient in this factor. The amount excreted after large doses was apparently of the same magnitude but was influenced by the amount of vitamin present in the tissues.

Close relationships between thiamine and several of the very

active hormones have been recognized for many years; thyr through its action to increase general metabolism, adds t normal requirements for the vitamin;[68] in insulin hypogly experiments (rats), thiamine increases sugar tolerance;[69] B_1 minosis causes hypertrophy of the adrenal cortex and a con tant increase in the cholesterol content of the blood. Ther certain suggested relationships between thiamine and ce minerals, notably zinc and manganese.[12a,12b,13,70] It has bee served that manganese ion, in certain concentrations, mar stimulates the carboxylase system.[17]

The biological assay and the thiochrome method are the cial" U.S.P. procedures[71] for determining the activity of thia containing substances. In the biological method the experim animals (rats) are carried through three stages:

(1) The depletion period, rats less than 30 days of age weighing not more than 50 grams, are maintained on the scribed diet, until symptoms of polyneuritis are evident, not m than 75 days.

(2) The preliminary period during which the diet supports nor development.

(3) The assay period, while on the same diet as in 2 the anin are given a single dose of standard or unknown adequate to p duce a curative effect enduring for not less than five or more th 15 days. In the thiochrome method the test material is diges with 0.1N sulfuric acid on a steam bath, after prescribed n tralization the enzyme (having diastatic and phosphoroly activity) is added, and after incubation the mixture is centrifug The supernatant liquid is transferred to a volumetric flask a diluted to 100 cc. An aliquot is passed through the "base change tube," subsequently eluted with hot acid potassi chloride. The thiamine is standard and unknown is oxidized thiochrome, the color is shaken out with butyl alcohol and typical fluorescence, 350 to 400 mμ, measured photometrically The colorimetric method proposed by Prebluda and McCollum,[74a] subsequently modified by Melnick and Field,[74b] has been used tensively and the results obtained compare favorably with valu obtained from bioassays.

It has been customary to regard animal assays as measu physiologically available nutrients but experience has proven they may not be always reliable when applied to human nutri

TABLE I. THIAMINE LOSSES CAUSED BY COOKING[a]

Food	Cooking Method	Per 100 Gm. Raw Food	Per 100 Gm. Cooked Food	Retained by Cooked Food	Dissolved in Cooking Liquid	Destroyed During Cooking
		I.U.[b]	I.U.[b]	%	%	%
Carrots	Pressure cooker	19	20	100	..	0
	Boiled	22	25	100	0	0
Potatoes	Baked	50	50	84	..	16
	Boiled (pared)	45	32	67	13	20
Spinach	Boiled	43	38	70	8	22
Peas, green	Simmered	150	124	80	11	9
	Simmered with soda	150	100	67	11	22
Beans, snap	Boiled	22	18	68	14	18
	Boiled with soda	22	11	41	Trace	59
Beans, Navy	Boiled	250	75	100	.	0
	Boiled with soda	250	80	100	..	0
Oats, rolled	Double boiler	200	33	100	..	0
Wheat, whole	Double boiler	180	36	100	0	0
	Baked (bread)	180	100	86	..	14
Pork loin, lean portion	Braised (*chop*)	485	550	85	..	15
	Roasted	455	400	57	Trace	43

[a] Data from Aughey,[41] E., and Daniel, E. P.: *J. Nutrition*, 19: 293, 1940.
[b] One U.S.P. (International) unit of vitamin B_1 = 3 μg of the crystalline hydrochloride, M.P. 246–247 °C.

Melnick et al.[75] have suggested a procedure for determining the availability of vitamins directly on human subjects. The principle of the procedure is based on a linear dose-response relationship established by feeding the vitamins in pure and most readily available form (solutions). With human subjects on an adequate diet, it has been established that excretion of water-soluble vitamins (thiamine, riboflavin, niacin, ascorbic acid) or their derivatives, is directly proportioned to the amount ingested.

The work of Baker, Wright and Drummond[76] in England, and of Jolliffe[31a] in this country indicates that the average diet of one hundred years ago had a much higher content of thiamine (vitamin B_1) than that of today. The usual methods of cooking, the decortication of cereals, the pasteurization of milk and the waste of cooking liquors all tend to reduce the actual intake of many of the vitamins. See Table I. Since the tissues of the human body have limited capacity for storage of the water-soluble B factors, it is essential that they be present regularly in the diet, or if lacking in the diet, that they be supplied by some other means, such as vitamin enriched foods, concentrates of natural foods and/or the synthetic factors. Although thiamine and most of the factors of the B complex are widely distributed in nature, space permits only mention of a few of the important dietary sources of thiamine. Cereals: notably those containing branny coats (brown rice, whole wheat bread, cracked wheat, oats, rye); lean pork; egg yolk; legumes (soy beans, dried pinto beans, kidney beans, green beans and green peas) are good sources of thiamine; of lower vitamin B_1 content: milk, lean beef, potatoes, cabbage.

The more liberal and optimal amounts of B_1 (National Research Council), needed to meet the demands of various age groups and different physiological states are shown in detail in Table II, page 242.

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Chapter 6

Riboflavin (Vitamin B_2)

Although it had long been known that the tissues of plants and animals contained water-soluble, greenish, fluorescent pigments, the substances received little or no attention from the chemist. Apparently the yellow pigment present in milk attracted some attention for Blyth[1] reported the isolation of lactoflavin (impure) in 1879. The nutrition studies of Osborne and Mendel[2] showed that milk contained a water-soluble accessory necessary for growth. The work of McCollum and Davis[3] indicated that it was not a lack of the fat-soluble accessory which was responsible for the failure of the experimental animals to grow and maintain good condition. In summary McCollum and Davis state: "The essential accessory aside from that carried by butter fat is present in water and in alcohol extracts of wheat embryo and egg yolk," and, "The accessory substance which is soluble in water and in alcohol is stable to heat." Later Emmett and McKim[4] demonstrated that an extract of rice polishings contained two vitamins, one which cured polyneuritis and one which produced weight. In these experiments[3,4] we have the earliest indications of the presence of two water soluble accessories (B_1 and B_2).

Real interest in these yellowish pigments was aroused by the work of Warburg and Christian[5a,5b,5c] on the yellow oxidation enzyme obtained from aqueous extracts of yeast. This study showed that the "yellow enzyme," through its easily reversible oxidation-reduction actions, serves as a transport between molecular oxygen and the substrate. Further work demonstrated that the "yellow enzyme" was present in all living cells. The "yellow enzyme" from yeast was also separated into two parts, one a protein, the other a pigment, and then showed that neither component was an active catalyst.[5] Theorell[6a,6b,6c] isolated the yellow oxidation enzyme in crystalline form and showed that the

riboflavin existed in the enzyme as a phosphoric acid ester,[6a] a nucleotide in which riboflavin is substituted for the usual purine complex. It is probable that the phosphoric acid is a connecting link between the protein and riboflavin components of the yellow enzyme.

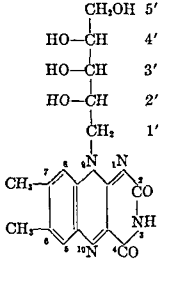
In 1933 Ellinger et al.,[7] Booher,[8] and Kuhn et al.,[9] published work which indicated a relationship and the probable identity of the water-soluble, green fluorescent pigments with the material isolated (in the form of concentrates) from liver, egg yolk, yeast etc., and commonly referred to as vitamin B_2 or G, and known to promote growth in the rat. Kuhn et al.[9] suggested the group name of "flavin" and since there were some differences in vitamin activity, it was assumed that there were differences in chemical structure, depending upon the source of the flavin. For some time the literature contained references to "ovoflavin," "heptoflavin," "lactoflavin," etc. Further work demonstrated that an identical flavin was obtained from each of the several natural products. The term "riboflavin" was proposed by the Council on Pharmacy and Chemistry[10] as the proper designation for lactoflavin, heptoflavin, ovoflavin and synthetic d-riboflavin, and for the then used "vitamin B_2" or "G."

Crystalline flavins have been isolated from a large number of plant and animal products; liver,[11] milk,[12] egg white,[13] kidney, egg yolk,[14] grasses,[15] dandelion blossoms.[16] In 1935 both Kuhn[17] and Karrer[18,19] (and their collaborators) published methods for synthesizing riboflavin. Although a large number of flavins have been prepared synthetically,[20,21] only riboflavin, the 5′-phosphoric acid ester and the flavin-adenine-dinucleotide possess the characteristic and complete vitamin activity. The following compounds are about one half as active as riboflavin, for the rat and lactic acid bacteria.[22]

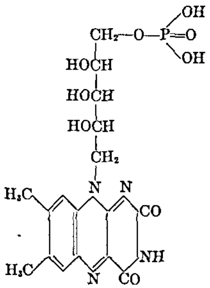
7-Methyl-9-(d,1′-ribityl)-isoalloxazine
6-Methyl-9-(d,1′-ribityl)-isoalloxazine
6-Ethyl-7-methyl-9-(d,1′-ribityl)-isoalloxazine

Pure synthetic riboflavin [6,7-dimethyl-9-(*d*,1′-ribityl)-isoalloxazine] occurs as fine, orange-yellow crystals and possesses a very bitter taste (melting point of about 280°C.—with decom-

position). Crystalline riboflavin is stable under ordinary storage conditions, although it is advisable to protect it from light. The substance is not destroyed by the commonly used concentration of acids, but is rapidly destroyed by alkalies, especially at elevated temperatures. It is soluble in water; 12 mg. per 100°C. at 27.5°C., only slightly soluble in alcohol, and insoluble in ether, chloroform, and benzene. The yellowish aqueous solutions show a marked greenish-yellow fluorescence in natural light which is intensified when examined under ultraviolet rays. The fluorescence is so intense under ultraviolet rays that this property can be used for estimating riboflavin.[23] If acid or neutral aqueous solutions of riboflavin are exposed to light the yellowish fluorescent material changes into a blue fluorescent substance, lumichrome; the change may go from yellow to green to blue, depending upon the amount transformed. If alkaline aqueous solutions are exposed to light, the chloroform-soluble lumiflavin is formed.



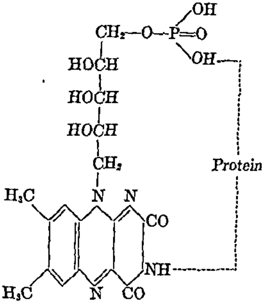
Riboflavin (B_2)



Riboflavin-5′-phosphate

It has been shown[24] that riboflavin is widely distributed in both plants and animals. Various yeasts, butyric acid bacteria (many anaerobic types of fermentation), mammalian liver, kidney, and heart contain large amounts of the vitamin. Riboflavin is an

important component of all enzyme systems which regulate cellular oxidation thus it plays an important role in general carbohydrate metabolism. According to Chase[25] free riboflavin plays an important part in the vision mechanism in the retina. By the action of light the vitamin is converted into a very sensitive "photo-compound." Under *anaerobic conditions* it is destroyed—in the presence of oxygen it is reconverted to riboflavin.



"Yellow Ferment"

A number of different enzyme systems, each containing riboflavin, are found in living tissues. Each system contains a specific protein (apoenzyme) and a prosthetic group (coenzyme) with riboflavin as a component of the latter. There are two coenzymes containing riboflavin, the mononucleotide (riboflavin-phosphate) and the dinucleotide (riboflavin-adenine-dinucleotide). By combining with different proteins, the 2 coenzymes form a variety of *enzyme systems, each capable of carrying out a specific reaction.* The enzymes, which act upon certain substances to remove hydrogen (with accompanying oxidation), are designated as dehydrogenases. Some of these are aerobic in nature and have the capacity to transfer hydrogen to some suitable acceptor, such as a flavoprotein. The dehydrogenases may be regarded as enzymes which inaugurate a series of reactions which ultimately result in the

formation of water. The phosphorylation of riboflavin is a common cellular reaction[26] and if this mechanism is blocked, as by removal of adrenal glands, no riboflavin is absorbed and animals (rats) cease growing.[27a,27b] The addition of the mononucleotide or the dinucleotide causes a resumption of growth. The levels of the dinucleotide in the blood cells, and riboflavin in the blood plasma (0.5 μg. per gram of blood) are quite constant.[26] Some riboflavin is excreted via the kidneys—the larger amount is found in the feces. While most of the vitamin is excreted in the free form, varying amounts of the phosphoric acid ester also appear in the urine. During periods of riboflavin deficiency there is none of the vitamin in the urine. An increase in the riboflavin intake of man leads to an increased urinary excretion.[28] On a balanced diet the normal adult excretes 500–800 μg. per day.[23] In addition to free riboflavin and the phosphoric ester, uroflavin is found in the urine. It has been shown by animal experiments[29,30] that there is an inverse relationship between the amount of protein in the diet and the urinary excretion of riboflavin. Rats maintained on a low protein diet excrete so much riboflavin that they are unable to keep a tissue level adequate for performance of function and die of riboflavin deficiency even though large doses of the vitamin are administered.

Sebrell and Onstott[31a,31b] showed that the dogs maintained on the blacktongue-producing diet would not survive unless riboflavin was added to the diet. Street and Cowgill[32] found that dogs maintained for 102 to 140 days on a diet deficient in riboflavin suffered a characteristic collapse and that prompt injection of the vitamin, 0.75 mg. per kilogram of body weight, resulted in immediate recovery. The authors found the addition of 25 micrograms of riboflavin (per day) to the deficient diet resulted in healthy maintenance of the animal over extended periods. Zimmerman et al.[33a,33b] maintained dogs on a balanced ration, deficient only in riboflavin (B_2), and produced a syndrome characterized by progressive muscular weakness, vomiting, diarrhea and fatal termination if the vitamin were withheld too long. These investigators reported extensive changes in the central nervous system involving, in varying degrees, most of the fiber tracts of the cord. Extension of this work confirmed the earlier findings with respect to

changes in the peripheral and central nervous system and it was found that dogs receiving adequate amounts of the vitamin did not develop any clinical or pathological signs and symptoms.[33b]

The literature contains many references to the eye lesions of experimental animals on B_2-deficient diets. Bessey and Wolbach[34] reported that vascularization of the cornea (rats) was an early and constant effect of riboflavin deficiency. Eckardt and Johnson[35] also noted marked keratitis of the cornea (rats) as a result of riboflavin deficiency. A considerable amount of experimental work (rats) indicates that a deficiency of riboflavin causes cataracts to develop in 70 to 80 per cent of the animals.[36,37] According to Day et al.[33] only a small amount (30 μg. per rat per week) of the vitamin is necessary to prevent cataract formation. The eye changes observed in flavin deficient rats are also observed in man. There may be itching, burning sensations and a mild photophobia; in instances of severe deficiency corneal opacity has been observed. In regard to the eye changes in man, Yudkin[39] attributes cataract formation to senility, local changes in the ocular tissues and general disturbances of metabolism. Yudkin believes it important to ascertain the role of vitamins in the maintenance of normal nutrition and health in middle aged persons, since deficiencies result in diminished vitality and the early onset of senility (the senile individual is predisposed to cataract).

As a result of feeding riboflavin (B_2) to rats at four distinct levels, Sherman and Ellis[40] found that the optimal requirements were much greater than the "necessary" intake. The enrichment of an already adequate diet with riboflavin (B_2) produced animals of greater vitality, an increased growth in the experimental animals and their offspring and a postponement of senility. György[41] administered riboflavin both parenterally and orally, to deficient rats and noted that the growth promoting effects were independent of the method of administration. Hussemann and Hetler[42] found that the rat required both thiamine (B_1) and riboflavin (B_2) for successful lactation and of the two substances, riboflavin appeared more important. Lepkovsky and Jukes[43] observed that rats maintained on diets deficient in riboflavin (G) grew but little; the hair fell out; the paws and faces were generally [illegible]dy and the eyes and eyelids often sw[illegible] shut. The [illegible] of 10 to

20 μg. of riboflavin to the diet caused immediate and marked improvement. Shaw and Phillips[44] demonstrated that riboflavin deficiency in the rat produced histopathological changes in certain of the endocrine glands and in the central nervous system. There exists an important relationship between riboflavin and the adrenal cortex, without the hormone(s) of the adrenal cortex the animal body is unable to phosphorylate the vitamin. Adrenalectomized rats will live and grow when fed riboflavin-5′-phosphoric acid, but promptly die when given riboflavin.[27a, 27b]

A deficiency of riboflavin causes one general symptom in all animal species, cessation of growth of all young. A total lack of vitamin causes prompt death of young and adults. Riboflavin deficient rats show an early atrophy of the testes, involution of the thymus, alopecia and, of special interest, loss of transparency of the lens[45] (cataract). Pigs[46, 47] require the vitamin for growth and general well-being.

Bethke et al.[48] noted that dried milk, alfalfa meal, autoclaved yeast, dried liver, etc., contained a substance which increased the hatchability of eggs. It was shown that the factor was water-soluble, soluble in alcohol and destroyed by heating in alkaline but not in acid solutions. Lepkovsky and Jukes[43] found that young chick and turkey poults require riboflavin for normal growth. Hegsted and Perry[49] found that riboflavin stimulated egg-production during the winter months. Riboflavin deficiency in breeding hens produced definite defects usually followed by death of the embryo. Chicks from eggs of hens receiving adequate amounts of the vitamin grew better and had more vigor than chicks from eggs of hens deficient in the vitamin.[50]

Snell and Strong[51] proved that riboflavin is indispensable for several lactic acid bacteria (*L. delbruckii*, *L. casei* and *L. casei acidi*). The *l*-araboflavin which is about one-third as active in promoting rat growth is poorly utilized by the lactic acid bacteria.

Owing to the fact that vitamin B_2 (or G) concentrates used up to 1937 contained several of the B factors, it is not surprising that the preliminary work in nutrition was full of misinterpretations. In the early studies on pellagra, Goldberger, Wheeler and Sydenstricker[52] noted that the various signs and symptoms of the syn-

drome indicated the presence of the least two separate nutritional factors. The clinical literature of 1933 to 1938 contained numerous reports from various parts of the world, in which nutritional deficiency syndromes were described and which included a discussion of the changes (typical symptoms of riboflavin deficiency) occurring in the skin, particularly about the angles of the mouth. Perusal of the literature on pellagra also reveals that ocular symptoms were frequently observed even in the earliest studies, and it is probable that some of the lesions described were due to riboflavin deficiency. Although not always present it would appear, from the standpoint of discomfort and disability, that the ocular lesions are the most important consequences of riboflavin deficiency. In areas where pellagra was endemic a large proportion of the children exhibited the characteristic ocular lesions of ariboflavinosis.[53]

With pure nicotinic acid (P-P factor) and riboflavin (B_2) available Sebrell and Butler[54] were able to clear up certain misconceptions by distinguishing symptoms of nicotinic acid deficiency in pellagra from symptoms or riboflavin deficiency (frequently but not always observed in pellagra). Eighteen women were placed on a diet deficient in riboflavin (Goldberger type) and within 94 to 130 days 10 of the group developed lesions of the lips. The clinical syndrome described by Sebrell and Butler[54] was characterized by an early pallor of the mucosa in the angles of the mouth and during the course of the changes, the lips became fiery red, shiny and superficially denuded along the line of closure. For these various changes the authors proposed the term "cheilosis." The syndrome included a scaly, greasy, desquamative lesion, with edema in nasolabial folds, in the vestibule of the nose and frequently around the eyelids and in the ears. Under the experimental conditions, small doses of riboflavin promptly alleviated the symptoms (not affected by relatively large doses of nicotinic acid). Jolliffe et al.[55] described lesions in 15 patients which were similar to but more advanced than the lesions experimentally produced by Sebrell and Butler.[54] All lesions improved following the administration of either natural or synthetic riboflavin. Subsequently, Vilter et al.[56] noted that the administration of riboflavin caused improvement in certain cases of pellagra, and Oden et al.[57]

observed three cases of riboflavin deficiency in private practice which were cured by administration of the vitamin. Spies et al.[58a,58b] noted that certain of their riboflavin-deficient patients gave a history of visual disturbances. Eye lesions observed were characterized by burning sensations, conjunctivitis, lacrimation and failing sight, some of which responded favorably to riboflavin therapy. Jolliffe et al.[55] also observed seborrheic excrescence located in the nasolabial folds. Kruse et al.[59] called attention to changes in the tongue, a specific type of glossitis characterized by flattened papillae (rather than atrophic) and a magenta red color (in contrast to the brick red tongue of pellagra), as a part of the syndrome of ariboflavinosis. Sebrell and co-workers[60] made extensive studies of the urinary excretion of riboflavin in two groups of women: (1) Ten women as experimental subjects; and (2) seven women maintained on the Institutional diet as controls. The basic diet of all contained 0.5 mg. of riboflavin per 2400 calories. The controls showed an average daily urinary excretion of 357 μg. of riboflavin; during the depletion period (131 to 254 days) the average daily urinary excretion of group I dropped to 77 μg. From these long term studies the authors estimate that 0.035 mg. of riboflavin per kilogram of body weight is not sufficient to meet adult daily requirements; the 0.06 mg. per kilogram of body weight is more than the adult requires, and concluded that the adult required about 3 milligrams of riboflavin per day. Williams et al.[61] maintained four female human subjects on a standard, and except for riboflavin, adequate diet for 288 days. The diet was appetizing but was restricted in that eggs, cheese, milk, organ meats, legumes and leafy vegetables were not allowed. The foods ingested contained 0.35 mg. of riboflavin per 1000 calories. At this level the only evidence of a deficiency was the progressive decrease in the excretion of the 2 mg. test dose. These investigators conclude that 0.5 mg. of riboflavin per 1000 calories represents the minimal daily requirement and 0.8 mg. per 1000 calories appears to be adequate for moderately active adults. Brewer et al.[62] studied the urinary excretions of riboflavin of 20 college students on self-selected diets with controlled vitamin intake and concluded that an intake of 1.3 to 1.5 mg. of riboflavin per day was adequate for women ingesting 2100 to 2300 calories.

McIntire et al.,[63a,63b] Schweigert et al.,[63c] Feaster et al.[64] and Rice and Robinson[65] studied the loss of various vitamins during the cooking (under standard conditions) of various types of meat and found that there was little or no loss (destruction, leaching, etc.) of riboflavin, a minimum of 80 per cent of the vitamin being retained in the cooked meats. It has also been shown that no significant loss of riboflavin occurs during the quick or slow freezing[66,67] of vegetables (commercial methods). Guerrant et al.[68a,68b] made an extensive study of the effects of commercial canning methods and found considerable loss (50 per cent) of riboflavin occurred during the canning of lima beans, spinach and alaska peas. Storage of canned vegetables at 30 to 42°F. was satisfactory for all foods and all vitamins included in the study. Storage at 85 to 110°F. resulted in marked losses of all vitamins (ascorbic acid, thiamine, riboflavin, pantothenic acid and carotene). Van Duyne et al.[69] studied the effects of home cooking on the riboflavin content of cabbage, peas, green beans and spinach. When cooked in a minimum amount of water for the shortest adequate time, the loss of the vitamin varied from 12 to 31 per cent, being greatest in peas and spinach. Sutherland et al.[70] compared home cooking methods with the large-scale Institutional procedures. Cabbage cooked in an open pan retained 42 per cent of the riboflavin, when cooked in steam jacketed kettle 58 per cent was retained. Open pan cooking of spinach resulted in 63 per cent loss of riboflavin, when cooked in a large kettle there was a 47 per cent loss of riboflavin. Holding the cooked vegetables on a steam table for 90 minutes did not cause any additional loss of riboflavin. Cheldelin et al.[71] studied vitamin losses during the home-type cooking of 30 common foods and found that the loss of riboflavin was greatest when cooking was done in the light. As much as 48 per cent of the vitamin was lost in the open frying of eggs, porkchops, sweet potatoes or in roasting meat (lamb, pork). These authors raise the question as to whether certain of the vitamins become "bound," rather than destroyed. Heller, McCay and Lyon[72] made an extensive survey of large-scale cookery (Navy Yard Cafeteria) and found that there were extensive losses of riboflavin, 22 to 45 per cent, during the preparation, cooking and holding of foods. Holmes et al.[73] found an average of 1.51 mg. of riboflavin per

liter of raw milk (32 samples). After carefully controlled pasteurization the same samples showed an average of 1.48 mg. per liter. Sullivan et al.[74] studied the vitamins present in 12 different types of cheese and found a fairly constant value for riboflavin, 4.0 to 8.3 μg. per gram.

There is definite evidence that the bacteria in the intestinal tract of man[75, 76, 77] and the rumen of certain animals[78a, 78b, 79] have the ability to synthesize several vitamins of the B group. However, there are so many variables affecting the extent of bacterial synthesis, and as a result the amount of any given vitamin provided by this means can never be determined with accuracy. As far as man and most animals are concerned, it is safer and easier to plan the diet so that it supplies the entire requirement for each vitamin.

Nutritional studies have shown that riboflavin is an essential nutrient for man, dog, pig, rat, duck and chick. Unless the food contains adequate amounts of the factor there is retardation of growth and impairment of general health, manifested by changes in the skin and finally by nervous system changes. Animals maintained on a riboflavin-deficient diet for appreciable intervals have been found to be in critical condition some days before death, an impending collapse which was usually not indicated by outward signs. Although present in all living cells, therefore in all tissues, riboflavin is not stored in large amounts nor can the normal tissue levels be greatly increased by administration of large doses.

While many factors may contribute to the daily requirement, it is generally accepted that mammalian needs are determined by body size and weight, metabolic activity, as well as the amount and nature of food ingested. According to Sebrell et al.,[60] man requires 2 to 3 mg. (30 to 42 μg. per kilo) of riboflavin daily. Cooperman et al.[80] observed that young monkeys developed symptoms of riboflavin deficiency on an intake of 12 μg. per day and placed the daily requirement of the young at about 25 μg. daily. The need of the adult monkey is probably not more than 15 to 20 μg. daily. Spector et al.[81] found that young dogs require at least 30 μg. per kilo per day for good growth and adequate regeneration of hemoglobin. The adult dog maintains normal weight and shows good regeneration of hemoglobin at 15

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μg. per kilo per day. Hens require 230 to 245 μg. per 100 grams of ration to ensure normal hatchability of eggs.[82a] Young chicks require much more for good growth.[82b] Turkeys require about 300 μg. per 100 grams of ration. Bacterial synthesis in the rumen of cattle and sheep provide adequate amounts for these species.

The riboflavin content of pure solutions may be determined by its characteristic fluorescence spectrum[23] at 565 mμ (pH 6.0). Van Duyne[83] modified and used this procedure in a study of tissue riboflavin after enzymatic hydrolysis. The rat growth test,[84a,84b,84c] the oldest and most used of methods of assaying has been replaced by the chick growth test (within certain limits the test is a linear function of the riboflavin administered) and both are rapidly giving way to the microbiological assay[85,86] using *Lactobacillus casei* as the assay organism. This method measures not only free riboflavin but also that combined (coenzymes). The most promising chemical test for detecting riboflavin deficiency, or the tissue saturation test, utilizes the measurement (by microbiological assay) of the vitamin excreted in the urine after administration of oral test dose. For the complete description of this procedure see reference (75) in the thiamine section.

Fortunately, riboflavin is generously distributed in plant and animal tissues and since it is one of the more stable vitamins excessive losses do not occur during handling, freezing, canning or cooking. Some of the richest food sources of riboflavin are liver, heart, eggs, lamb, chicken (dark meat), bacon, pork, veal, beef, whole wheat bread, oatmeal, asparagus, peas, beets and peanuts.

The more liberal and optimal amounts of B_2 (National Research Council) needed to meet the demands of various age groups and *different physiological states are shown* in detail in Table II, page 242.

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of a factor in the B complex, other than the then recognized B_1 and B_2 (G), also that the various substances effective in curing canine blacktongue were equally effective in curing human pellagra. Aykroyd and Roscoe[13] also observed that the canine and human factors occurred together.

Niacinamide is a normal cell substance and a growth factor for plants and microorganisms. It is probable that the coenzyme rôle in the reversible reduction-oxidation reactions is its principal activity.

The numerous enzyme systems present in all plant and animal cells contain certain dehydrogenases which are concerned with various dehydrogenation reactions as well as the transport of hydrogen. Two coenzymes, of this class of dehydrogenases, containing nicotinamide are known: Coenzyme I (codehydrogenase I) and Coenzyme II (codehydrogenase II). Many apoenzymes (proteins) combine with each of the coenzymes and thus enter into a large number of reducing and oxidizing reactions. Probably all dehydrogenation reactions are reversible, equilibrium constants have been determined for many *in vitro* reactions catalyzed by the coenzymes. In living tissues there is no equilibrium since the reaction products do not accumulate.

Coenzyme I (Codehydrogenase I, Cozymase, Coferment) is found in all plant and animal tissues (cells) wherever carbohydrates are metabolized. Heart muscle contains a high concentration,[14] 40 mg. per 100 Gms.; red blood cells and yeast cells[15] are also especially rich sources of coenzyme I. During the dehydrogenation reactions, the coenzyme is reduced to dihydrocoenzyme; with another apoenzyme (protein) the reduced compounds are oxidized. The muscle tissues of all animals contain a fairly constant ratio of coenzyme (55 to 65 per cent) to the reduced coenzyme (35 to 45 per cent).[16]

Coenzyme I is colorless, soluble in water, insoluble in the common organic solvents, and exhibits a characteristic absorption spectrum with a maximum at 260 mμ.[17] The coenzyme is stable in acid solutions,[18] unstable in alkaline media.[19] The reduced compound is acid labile and alkali stable.[20] The coenzyme I is optically active. Hydrolysis of the dinucleotide (coenzyme I) yields: adenine,[21] nicotinamide,[12b,23] and 2 mols of d-ribose-

Chapter 7

*Niacin, Niacinamide**

(Nicotinic Acid, Nicotinic Acid Amide)

In the search for the antineuritic vitamin (B_1) both Funk[1a,1b] and Suzuki[2] isolated nicotinic acid (from yeast or rice bran) and tested it on polyneuritic pigeons. However, each investigator failed to ascertain the true vitamin properties of the material isolated.

Niacin (Nicotinic acid) was synthesized in 1873 by Huber[3] and by Weidel.[4] The compound readily crystallizes from water or alcohol. The needle-like crystals melt at 235.5 to 236.6°C.[5] and the compound exhibits a typical absorption spectrum at 385 mμ.[6] Niacinamide (nicotinic acid amide) crystallizes in needles from benzene and has a melting point of 129°C. Niacin and niacinamide are readily soluble in water, alcohol and glycerine. Aqueous solutions are stable and may be heat sterilized.[7]

Pellagra was described as a human disease by Casal as early as 1735, and its widespread occurrence was noted by 1907. By dietary restrictions, Chittenden and Underhill[8] produced pathological changes in dogs which were designated as "canine black tongue." Goldberger and Tanner[9] coined the phrase "pellagra-preventive (P.P.) factor" to indicate the substance which was necessary to prevent human pellagra. Employing dietary restrictions Goldberger and Lillie[10] were able to produce a pellagra-like syndrome in rats and, later, Norris and Ringrose[11] produced pellagra-like lesions in chicks. The work of Goldberger and coworkers,[12a,12b,12c] contained in more than 30 papers, demonstrated that human pellagra was a deficiency disease caused by the lack

* Press release, Federal Security Agency, Jan. 19, 1942. Federal Security Administrator announced acceptance of the recommendation of Committee on Foods and Nutrition, National Research Council that the term "Niacin" and "Niacinamide" be adopted as synonyms for the vitamin substances scientifically designated as "Nicotinic acid" and "Nicotinic acid amide," respectively.

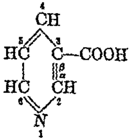
nicotinamide with a pentavalent ring-nitrogen yield dihydro-compounds with the same absorption spectrum as the reduced coenzyme. In the coenzymes the ring-nitrogen of nicotinamide is bound in such a manner that reduction results in tertiary nitrogen.

The animal and plant foods supply the normal dietary intake of niacin, mostly in the form of coenzymes. It is not known whether the enzyme complexes are hydrolysed in the intestinal tract prior to absorption or whether they can be absorbed unchanged. Nicotinic acid and the amide are readily absorbed unchanged and transported in the blood (serum) which maintains a certain, essential level. The red blood cells contain no free niacinamide but relatively large amounts of the coenzymes. As previously stated nicotinamide, in the form of coenzymes, is present in all living cells but there are no special storage organs. Niacin deficiency, in man, dog and pig, results in a marked decrease in the coenzyme content of the liver and the muscles,[29,30,31] but there is no significant concomitant decrease in the coenzyme content of the erythrocytes.[32]

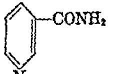
Under normal conditions man excretes niacin and its end-products through the urine, the total excretion depending upon the intake. According to Harris and Raymond,[33] on the average diet there is a daily urinary excretion of 4 to 5 mg. Sarett et al.[34] found 9 to 13 mg. of trigonelline per day, on a rigorously controlled diet. Melnick et al.[35] studied the niacin excretion of 11 well nourished human subjects and found that the basal 24 hour excretion values ranged from 1.7 to 29.3 mg. The urine contains a small amount of free substance together with relatively large amounts of nicotinuric acid and trigonelline.[36] Coenzymes are not excreted.[37,38] In pellagra, and during periods of general anorexia, there is a marked decrease in the excretion of niacin and its metabolic derivatives. Dogs excrete no niacin or niacinamide in the urine, only nicotinuric acid and trigonelline.[36,39] Rabbits are unable to convert niacin into trigonelline.[36]

Koehn and Elvehjem[40] demonstrated that a purified liver concentrate completely cured the symptoms of canine blacktongue. These authors also stated that the liver concentrate cured the symptoms of chick pellagra (dermatitis); however, this was shown

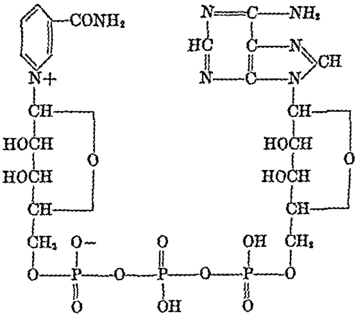
phosphoric-acid. In one of the papers from von Euler's[24] laboratory it was stated that a purified cozymase preparation contained a basic component which *yielded nicotinic acid amide* upon hydrolysis. Holiday[25] studied spectra of concentrates of Staphylococcal cultures and was able to demonstrate the presence of considerable amounts (1.5 per cent) of nicotinic acid.



Niacin



Niacinamide



Coenzyme I, Cozymase

It has been shown by in vitro experiments that certain blood constituents, the nucleated white cells of lymphoid and myeloid series, can convert niacin and niacinamide into coenzymes I and II. According to Warburg et al.[26] the nicotinamide is responsible for the reversible reduction-oxidation action of coenzymes I and II. Experiments[27,28] *indicate that only those* derivatives of

was not curative; 0.2 mgm. per kilogram per day was slowly curative and that 0.5 mgm. per kilogram gave rapid and complete cure of all blacktongue symptoms. It was pointed out that the Goldberger blacktongue-producing diet is also deficient in vitamin B_1 (thiamine) and B_2 (riboflavin). If dogs are used repeatedly in testing, it is necessary to add these components to the diet in order to obtain complete cures. In extended tests with nicotinic acid on dogs, Margolis et al.[53] emphasized the ineffectiveness of the 0.1 mgm. per kilogram dose, noted that the 0.2 mgm. per kilogram daily dose gave protection but little gain in weight, and selected 0.5 mgm. per kilogram as the minimum dose for a prompt and complete cure of blacktongue (return of appetite, gain in weight and disappearance of typical mouth lesions).

At the time nicotinic acid (amide) was isolated and shown to be effective in canine blacktongue, it was generally accepted that the disease in dogs was the analog of pellagra in man. The symptomatology and pathologic lesions were similar; every substance that prevented one was effective in the other, and a substance ineffective in one had no value in the other. It was, therefore, natural and obvious that nicotinic acid and the amide should be tried in the treatment of the human disease. In November 1937, Fouts et al.[54] reported that nicotinic acid caused prompt improvement in lesions of pellagra and that the new vitamin was equal in effect to liver filtrate, except that the dermatitis required more time for complete disappearance. Harris[55] noted that although nicotinic acid promptly cured certain lesions of pellagra, it was in all probability not the sole deficiency in many cases of pellagra. Spies[56] reported that 15 pellagrins had a prompt remission of the glossitis, stomatitis, ptyalism, vaginitis, urethritis and proctitis following administration of nicotinic acid, 100 mg. five times per day by mouth (three cases were also treated parenterally). In a subsequent paper Spies et al.[57] again commented upon the prompt cure of lesions of the mucous membranes. It was also stated that there was frequently a progression of the neurologic involvement (peripheral neuritis) and that certain cases of glossitis did not respond during the period of nicotinic acid therapy. The peripheral neuritis usually disappeared promptly when thiamine (B_1) was given. It was later noted[58, 59, 60]

by Dann[41] to be due to the presence of other B factors present in the liver concentrate. At this time Dann postulated, and correctly so as subsequent events showed, a *separate factor* was responsible for the cure of the chick dermatitis. To Elvehjem and his associates[42] goes the credit for the identification of *nicotinic acid* amide as the anti-blacktongue factor. It was shown that both the acid and the amide were effective in curing canine blacktongue and in maintaining dogs in a normal condition while fed a blacktongue-producing diet. In 1937 Elvehjem et al.[43] showed that the nicotinic acid amide isolated from liver and that prepared commercially from the alkaloid *nicotine* were equally effective in curing canine blacktongue. The authors, suggested that niacin was probably a part of an enzyme system and involved in oxygen transfer from blood to tissues. It was also pointed out that many animal species could not synthesize the substance and that it, therefore, had to be provided in the food.

Soon there appeared in the literature a number of reports confirming the effectiveness of nicotinic acid in blacktongue[44] and pellagra.[45] There soon followed the testing of other pyridine derivatives.[46,47] Woolley et al.[48] tested the anti-blacktongue activity of 19 pyridine derivatives other than nicotinic acid and amide) and found *five of them (ethyl nicotinate, nicotinic acid* N-methyl amide, nicotinic acid N-diethylamide, nicotinuric acid, nicotinamide glucosidoiodide) were equal in effectiveness in the cure of the symptoms of canine blacktongue. In addition to *niacin and niacinamide,* Vilter *and* Spies *et* al.[49] showed the N-methyl-amide and the N-diethyl-amide of nicotinic acid, the ethyl-nicotinate and nicotinuric acid are effective in the treatment of human pellagra. The ring-nitrogen must be unsubstituted since trigonelline is inactive in man. A number of other compounds (quinolinic acid, pyrazine-carboxylic acid, etc.) were active in human pellagra[50] but under their experimental conditions, ineffective in canine blacktongue.[47,48]

Sebrell et al.[51] found that 3.0 mgms. of nicotinic acid given twice per week was hardly sufficient and that 10 mgms. given twice per week was adequate to prevent the development of blacktongue *symptoms over* a period of six months. Smith and Smith[52] found that 0.1 mgm. of nicotinic acid per kilogram daily for ten days

diseases, cerebral arteriosclerosis.) On the basis of clinical work, Jolliffe et al.[62] suggested that the encephalopathic syndrome of alcoholism is the resultant of an acute nicotinic acid deficiency. The best treatment of this group includes parenteral fluids and nicotinic acid, together with supplements of the entire B complex. In order to prevent progression of the central nervous system involvements, Wortis and Jolliffe[63] suggest intensive therapy with thiamine hydrochloride, nicotinic acid and B complex.

Since deficiency diseases, like bacterial diseases, vary in severity, it is not strange that the dosage of nicotinic acid should be difficult to establish. Ruffin and Smith[64] found that the parenteral administration of 1.5 mgm. per kilogram (105 mgms. for 150 lb. person) per day was highly effective but that larger peroral doses were required. From clinical observations a survey of the diets of pellagrins, and the treatment of nearly 1000 patients with vitamin deficiencies, Spies, Vilter and Ashe[65] concluded that the average pellagrin was effectively treated by the peroral administration of 500 mgms. (10 doses of 50 mgms. each) of nicotinic acid (or amide). The daily parenteral dose, dissolved in sterile saline, should be given in divided doses.

The clinical use of nicotinic acid produces, in many patients, a considerable flushing of the face, neck and extremities as well as some disturbance of pulse rate and intensity of heart beat. Strangely, the substitution of an amino (NH_2) group for the H in the carboxyl group does away with the circulatory disturbances and, for this reason, nicotinic acid amide has largely replaced the acid. The usual clinical doses of this substance rarely produce any disturbance.

Although it is certain that all living tissues (cells) require niacin, there is little or no data to actually indicate the requirements of man, various animals and microorganisms. It has been shown that man, monkey,[66] dog[67,68] and pig[69] require an external supply of the vitamin. Previous work indicates that tryptophane is an important precursor of niacin synthesis and that the niacin deficiency syndrome is produced when the diet is lacking in preformed niacin and in tryptophane. Hundley[70] produced niacin deficiency in rats by feeding a high carbohydrate diet, low in casein and supplemented with other tryptophane-deficient pro-

that nicotinic acid rapidly relieved the mental symptoms and al agreed that the dermal lesions were the slowest to respond (7 t 10 days). Thus thiamine and riboflavin are required in additio to niacin for the complete treatment of pellagra. The clinica reports on the treatment of pellagra with nicotinic acid (or amide are many and come from all parts of the world. Furthermore and more important, the use of the vitamin as a prophylacti agent has been given a definite place in modern medicine.[60,61]

Characteristic lesions of pellagra are frequently observed i early childhood, especially in areas where pellagra is endemic The clinical signs and symptoms of alcoholic pellagra are es sentially those observed in endemic pellagra and the response t treatment is the same in both types. There are no definit symptoms by which the physician can determine mild niaci deficiency. To establish niacin deficiency it is necessary to de termine *blood levels and urinary excretion. Porphyrins* appea in the urine during the early stages of niacin deficiency.

Although dermatitis is fairly common in "alcoholic" pellagra it is not as characteristic a symptom as in the endemic form of th disease. Stomatitis is characteristic and frequently it is th earliest symptom to be observed in the alcoholic type. Th tongue, lips, gums, palate and mucus membranes are frequentl the sites of a fiery red process which is followed by ulceratio and infection by anaerobic organisms. The generalized stoma titis may be so severe that it is impossible to ingest solid food thus contributing to already present deficiencies. Gastroin testinal disturbances are common in alcoholics; diarrhea i serious and requires immediate and intensive treatment (paren teral preferable) with nicotinic acid.

Mental changes are noted in nearly every severe case of pellagra in typical alcoholic pellagra the patient is confused, hallucination and fear alternate, and in severe cases of alcoholic pellagr there frequently is produced a very definite and complete en cephalopathic picture—a condition almost invariably fatal. Th clinical picture is fairly well defined and includes clouding of consciousness, cogwheel rigidities of the extremities and un controllable sucking and grasping reflexes. (Similar manifesta tions are observed in delirium tremens, delirium of infectious

concluded that it was either synthesized in the tissues or was synthesized in the rumen by microorganisms. McElroy and Goss[82a,82b] reported that four members of the B complex (thiamine, riboflavin, pantothenic acid and pyridine) were synthesized by bacteria in the rumen of sheep. The niacin requirement of the rat has been extensively studied[68,70,83,84] and, although it is certain that the rat requires niacin, it seems that microorganisms present in the intestinal tract synthesize and supply the need.[85] It is also evident that cattle and sheep require the vitamin but each of these species provides its own needs through bacterial synthesis[80 81,82a,82b] (occurring in the rumen). Sarett and Perlzweig[83] showed that tryptophane may be used to supplement diets inadequate for rat growth. Mickelson et al.[86] showed that nicotinic acid and amide did not cure chick dermatitis and had no effect upon the growth of chicks maintained on a modified Goldberger diet. Several investigators have shown that nicotinic acid is an essential nutrient for the monkey.[66,87,88]

In 1937 Mueller[89] showed that a substance isolated from liver and identified as nicotinic acid exerted a striking effect upon the growth of the diphtheria bacillus. Knight and McIlwain[90] pointed out that thiamine (B_1) and nicotinic acid are specific requirements for the growth of *Staphylococcus aureus*. Snell et al.[91] demonstrated that niacin is an essential growth requirement for at least two species of lactic acid bacteria. The list has been rapidly extended to show that many of the common microorganisms require this vitamin for growth.

Several chemical methods, involving colorimetry, have been described in the literature, the most used being the procedure utilizing the cyanogen bromide-aniline reaction with the pyridine ring.[92,93,94,95] Snell and Wright[96] have developed a microbiological method of assay which consists in a measure of the lactic acid produced by *Lactobacillus arabinosis*. The physiological availability procedure of Melnick et al. (see thiamine reference, No. 75) may be used in human studies with niacin.

The daily requirements of man vary from 10 to 20 mg. per day depending on age, activity and physiological state. According to Harris[66] the monkey requires about 5 mg. per day. Dogs require about 0.25 mg. per kilo body weight for complete protection[68]

tein material (cystine). The data show that, when tryptophane is the principal factor in limiting the growth of young rats, there is a marked reduction in niacin synthesis, a fall in tissue level, and a diminished excretion of N′-Methylnicotinamide. The data also confirm the work of Singal, et al.,[71] Rosen et al.,[72] in establishing tryptophane as the dietary precursor of niacin. recent work of Ellinger and associates[73,74] definitely indi bacterial synthesis of niacin in the intestinal tract of man. hibition of bacterial activity in the intestinal tract by su guanidine or sulfasuxidine, resulted in a pronounced drop in excretion of N′-Methylnicotinamide. Najjar et al.[75] also demo strated bacterial synthesis of niacin in the intestinal tract of ma Human subjects placed on a diet very low in niacin, 1.5 to 2. mg. continued to excrete large amounts of N-Methylnicotinamide, as much as when the daily diet furnished 20 to 30 mg. of niacin-amide.

In 1935 Miller and Rhoads[76] and in 1937 Birch et al.[77] demonstrated that swine maintained on blacktongue diets developed lesions of the mouth and suffered from general nutritional failure. Birch felt that deficiency of some factor of the B complex, other than lactoflavin, was the cause of the symptoms. Chick et al.[78] demonstrated that the deficiency symptoms in swine were cured by administering nicotinic acid and stated that the experimental maize diet required only nicotinic acid to make it adequate for rearing pigs. Wintrobe et al.[79] found that when young pigs were given a high protein diet (26.1 per cent casein) plus thiamine, riboflavin, pyridoxine, pantothenic acid, inositol and p-aminobenzoic acid, but no niacin, there were no signs of niacin deficiency. Young pigs placed on a low protein diet (10 per cent casein) plus the same vitamin supplements, but no niacin, rapidly developed a deficiency syndrome; impaired growth, poor appetite, diarrhea, severe anemia and a consistent reduction in the urinary excretion of niacin derivatives. This work suggests a close relationship between protein and niacin requirement.

Pearson et al.[80] found that lambs develop normally on a diet which produces canine blacktongue and pellagra-like lesions in swine. Winegar et al.[81] found that sheep, maintained on a diet deficient in nicotinic acid, continued to excrete the substance and

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against blacktongue and for gain in weight, 0.5 mg. per kilo offers greater safety.

Excellent food sources of niacin (3,000 to 12,000 μg. per 100 Gms.): liver, halibut, salmon, heart, beef round, veal, bacon, chicken; good sources are (1,000 to 3,000 μg. per 100 Gm.) peas, potatoes, lima beans, turnips, grapefruit, whole wheat bread.[97,98] Camembert and Roquefort cheese are also good sources of niacin.[99] The heat stability of niacin leads to satisfactory retention during the cooking of meats [100,101] and most vegetables.[102] Poor handling, excessive blanching, or long holding on a steam table (careless handling in large scale cooking) may cause as much as 60 per cent loss of niacin.[103] See Table II, page 242, for suggested daily requirements.

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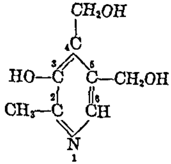
Chapter 8

Pyridoxine (Vitamin B_6)

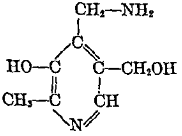
It is quite certain that vitamin B_6 was isolated from rice polishings as early as 1932. However, the vitamin properties were not recognized. In 1926 Goldberger and Lillie[1] reported that rats, maintained on a diet deficient in vitamin B_2 (at that date "B_2" was a mixture of several factors), developed a characteristic dermatitis, subsequently to be known as "rat acrodynia."

During 1926 to 1939 the substance we now know as vitamin B_6 was referred to under several names: anti-acrodynia factor; rat anti-dermatitis factor; yeast eluate factor;[2] factor I of Lepkovsky;[3] factor Y of Chick;[4] vitamin H of Hogan-Richardson,[5] and Booher;[6] also the complementary factor.[7] Between February and May of 1938, no less than four widely separated groups[8,9,10,11a,11b] of investigators announced the isolation of crystalline B_6 and two groups[12a,12b,12c,13,14] working independently, determined the chemical structure of the substance. Shortly thereafter the complete synthesis of vitamin B_6 was reported.[15,16] With the use of both rat-prophylactic and rat-curative tests, it was found that the pure natural and the pure synthetic vitamin B_6 were identical in their chemical and physiological properties.[17]

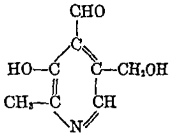
Pyridoxine hydrochloride (B_6), the form of the vitamin in commercial use, is a white, odorless powder with a salty taste. The melting point is 204–206°C.,[11b,13] with decomposition, the compound is optically inactive. Vitamin B_6 hydrochloride is readily soluble in water (1 Gm. in 4.5 cc.) and about 1 Gm. per 90 cc. of alcohol. The aqueous solutions, pH of 3.2, are heat stable and may be sterilized by autoclaving (120°C.). The vitamin is light sensitive. The free base melts at 160°C.,[13] is readily soluble in water, alcohol and acetone and reacts easily with various acids to form salts.



Pyridoxine (B_6)



PYRIDOXAMINE
2-Methyl-3-hydroxy-4-aminomethyl 5-hydroxymethylpyridine



PYRIDOXAL
2-Methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine

Credit is due György[18,19] for an early step in the unraveling of the B-complex; separating the "B_2 complex" into (lactoflavin), vitamin B_2 and B_6, the rat anti-dermatitis factor or rat pellagra-preventive factor. In a subsequent paper Birch et al.[20] differentiated the effects of lactoflavin from the new factor, B_6, and subsequently were the first to indicate the specific differences between the pellagra-preventive ("P-P") factor, lactoflavin (B_2), and the rat anti-dermatitis-factor (B_6). The term "rat acrodynia" was then proposed to describe the specific dermatitis in rats caused by a deficient intake of vitamin B_6. In 1938 Dann and SubbaRow[21] presented data to show that the administration of nicotinic acid ("P-P" factor) did not prevent chick dermatitis (pellagra) nor did it cure the rat dermatitis described by György. These investigators confirmed the earlier report of Elvehjem et al.[22] that nicotinic acid cured blacktongue in dogs and stated that the data then available (1938) clearly indicated the existence of four sharply defined components of the "B_2 complex"; riboflavin, nicotinic acid, vitamin B_6 and the "filtrate-factor" (Factor II of Lepkovsky).

Oleson et al.[23] described four syndromes produced in rats when the diet was low in the B complex (but adequate in thiamine, riboflavin, nicotinic acid and choline): acrodynia, hemorrhagic disease, "spectacle eyes" and paralysis. They found crystalline B_6 was very effective in curing the acrodynia but ineffective for the remaining syndromes. György and Goldblatt[24] observed diverse pathological changes in livers of rats kept on a diet deficient in the B complex (though adequate in thiamine, riboflavin and B_6), and since yeast and certain yeast extracts prevented the various lesions, they were assumed to be of nutritional origin and related to a deficiency of some component of the B-complex. Acrodynia in rats, produced by dietary methods, was improved by the addition of ten μg. per day of crystalline B_6, but in many instances the rats developed severe skin lesions (differing from those amenable to B_6 therapy) and frequently died from deficiency disease.[25] Antopol and Unna[26] produced a number of gross changes in rats fed a diet deficient in B_6, most striking of which were edema and ulcerations about the ears, paws and snout and changes in the hair matrix. A single dose of 100 μg. of crystalline B_6 resulted in rapid improvement, the edema being the first symptom to disappear.

The work of Birch,[27] Halliday,[28] and Schneider et al.[29] suggested a relationship between the vitamin and the metabolism of fatty acids and for some time it was thought that certain essential unsaturated fatty acids could protect the rat from developing the characteristic dermatitis produced in rats by a B_6-deficient diet. Although the dermatitis produced by a lack of B_6 or the essential unsaturated fatty acids is very similar, the B_6 dermatitis can be differentiated from the fatty acid syndrome by the edema which results only from the vitamin deficiency. In addition to the symmetrical dermatitis, young animals cease growing[30,31] and most animals (dogs, pigs, rats) exhibit epileptiform seizures.[30,31,32,33] Degeneration of cardiac and striated muscles as well as extensive pathological changes in the central nervous system have been observed. B_6-deficient rats also show marked changes in the sexual organs. Dogs show all of the above changes and, in addition, develop a hypochromic, microcytic anemia.[31,34,35]

Lepkovsky and Nielsen[36] noted that B_6-deficient rats excreted a

compound which was converted by ferric ammonium sulfate into a green pigment. Excretion of this substance stopped within a few hours after the administration of B_6. In a subsequent paper Lepkovsky et al.[37] reported that the urine of B_6-deficient rats contained an abundance of xanthurenic acid, a substance associated with protein (tryptophane) metabolism. It was also pointed out that the dog, deficient in B_6, and the rat, deficient in pantothenic acid, excretes little or no xanthurenic in the urine.

Very little is known concerning plant requirements for vitamin B_6. Möller[38] showed that certain B complex concentrates and later crystalline B_6 ("Adermin") were necessary for certain lactic acid bacteria; Hutchings and Woolley[39] found that some strains of hemolytic streptococci required the vitamin; Vilter and Spies[40] showed that the vitamin was necessary for *Staphylococcus albus;* Schultz et al.,[41] also Eakin and Williams[42] found that vitamin B_6 was a growth promoting factor for yeast. McElroy and Goss[43] proved that bacteria in the rumen of the sheep are able to synthesize the specie requirements for vitamin B_6.

Snell et al.[44] proved conclusively that the high B_6 activity of certain tissue extracts in promoting the growth of *Streptococcus lactis*, on a pyridoxine-free medium was due to the presence of an active metabolite which was formed from pyridoxine by the organism. The evidence indicated that change in the pyridoxine molecule took place prior to utilization. In a subsequent paper Snell[45] reported that the increased activity for *S. lactis* could be obtained either by amination or oxidation of pyridoxine and suggested that one of the products was an amine and the other an aldehyde. Harris et al.[46] verified the structures suggested by Snell and synthesized the two compounds, pyridoxamine and pyridoxal. Snell and Rannefeld[47] compared the growth-promoting properties of pyridoxine, pyridoxamine and pyridoxal on seventeen different organisms. The data demonstrated that pyridoxamine and pyridoxal were as active, or more active, than pyridoxine for fifteen of the organisms included in the study. This work shows that when ascertaining the B_6 activity of various substances, it is important to select an organism which reacts about the same to each of the three substances. Pyridoxamine [illegible] and are rapidly destroyed by

The requirements for pyridoxine are dependent to a large extent upon the composition of the diet. Some amino acids (tryptophane, methionine) show growth-depressing effects on B_6-deficient rats, mice and dogs. The inclusion of carbohydrate in the diet of experimental animals accelerates bacterial synthesis within the intestinal tract and thus provides enough B_6 for growth. Both pyridoxamine and pyridoxal are subject to destruction by bacteria present in the intestinal tract.

Jukes[48] showed that chicks deprived of B_6 grew slowly, had a poor appetite, exhibited poor utilization of ingested food and, in many instances, developed spasmodic convulsions. Later, Lepkovsky and Kratzer[49] reported that a B_6-deficiency in chicks resulted in: (1) excitability; (2) jerky, spastic movements; and (3) convulsions. These writers point out that before convulsions appear, the basal ration must be complete with respect to all other vitamin requirements. They also emphasized the complete absence of dermatitis. According to Briggs et al.,[50] young chicks require 275 to 300 μg. of crystalline B_6 per 100 Gm. of ration as a minimum level for good growth. Bauernfeind and Norris[51] reported that vitamin B_6 is required by domestic fowl for reproduction and hatchability of eggs. Pyridoxine deficiency in monkeys is characterized by: failure to grow; various and severe blood dyscrasias; and ataxia.[52] The work of Wintrobe et al.[33] indicates that in the pig, as in the dog, pyridoxine plays an important rôle in blood formation, and especially in iron metabolism. As the anemia of B_6 deficiency develops, there is an increase in the iron content of the plasma and marked hemosiderosis of the tissues. The rapidity of erythrocyte formation, following the administration of B_6, suggests that the vitamin is concerned with the utilization of iron and hemoglobin production.

Spies et al.[53] reported that following the intravenous injection of 50 mg. of pure B_6, the average urinary excretion in normal persons averaged 7.9 per cent. Nine ambulatory patients, all with clinical evidence of pellagra, beriberi or ariboflavinosis, excreted a maximum of 1.5 per cent of the 50 mg. test dose. Flexner and Chassin[54] studied the urinary excretion of pyridoxine by patients maintained on standard, balanced diets. In one hour 12 of 14 subjects, under 50 years of age, excreted 8.4 per cent of the 50 mg.

test dose. Thirty two patients, over 50 years of age, showed an average excretion of 7.2 per cent. Ten patients, between 5 and 15 years, excreted an average of 21.3 per cent of the 50 mg. test dose. This work suggests that the test dose should be based on body weight and emphasizes the importance of knowing the renal status of all persons treated.

Scudi et al.[55] used a colorimetric method, based on the indophenol reaction, to study the urinary excretion of pyridoxine in man and in the dog. The short interval between the oral administration of a test dose and the appearance of the vitamin in the urine is indicative of rapid absorption. Dogs excreted about 20 per cent of the test dose within six hours. Normal men excreted 7.6 per cent of the 100 mg. test dose within four hours.

In the early work with vitamin B_6 it was customary to use either the rat growth method or the cure of rat acrodynia as a means of determining the vitamin B_6 content of various biological products and foods used by man and animals. Clarke et al.[56] carefully studied two methods, each of which use the rat as the test animal. The one involving the cure of the typical acrodynia is open to objections; (1) the effects of other deficiencies on the occurrence and cure of the dermatitis, (2) marked variation in the severity of the dermatitis, and (3) the personal element involved in interpreting the extent and degree of cure of the dermatitis is too great. Clarke et al.[56] observed a satisfactory relationship between graded doses of the vitamin and gain in body weight. Atkin et al.[57] have proposed a microbiological method of assaying B_6 preparations using yeast, strain 4228, as the organism. Sarma et al.[58] point out that the microbiological assays measure the naturally occurring pyridoxine, pyridoxal and pyridoxamine and hence do not indicate the B_6 content of natural foodstuffs. Using a more nearly synthetic rat ration, a number of natural products were assayed. In general the values obtained by the rat assay are lower than those obtained by the microbiological assays. Hochberg et al.[59a] developed a chemical method, colorimetric, based on the coupling of the vitamin with 2,6-dichloroquinonechloroimide, to form a blue pigment. Maximum color is developed within 60 seconds. A subsequent report[59b] includes modifications and precautions for use of the

method in determining pyridoxine (activity) in biological and pharmaceutical materials.

The physiological action of vitamin B_6 in man and animals remains obscure. The free vitamin, but not the protein bound, is readily absorbed from the intestinal tract. There is no known storage organ. The vitamin is excreted in the urine, is found in milk and it is thought that the administration of excessive amounts results in large-scale destruction. Pyridoxine is found widely distributed in plant and animal tissues. Rice polishings, the branny coats of wheat and rice, wheat germ, corn germ are rich sources.[29,60] Mammalian liver, egg yolk, milk, and lettuce are good food sources.[61] Very little of the vitamin occurs free in either plant or animal tissues.[11a,11b,62] Most of it is protein-bound[11a] although some is apparently bound by starches.[32] The vitamin is readily released from such complexes by heat[11a,32] and by enzymatic hydrolysis.[62] Unless vegetables and seeds are cooked, animals (rats) are unable to utilize the full amount of B_6 present.

Ives and coworkers[63] determined the pyridoxine content of 101 samples of canned foods used commonly by the average American family. They report as "excellent" sources (60 to 130 μg. per 100 Gm.): salmon, tomatoes, yellow corn and spinach. "Good" sources (30 to 46 μg. per 100 Gm.): peas, green asparagus and green beans. There are no data available to indicate the human requirement for vitamin B_6.

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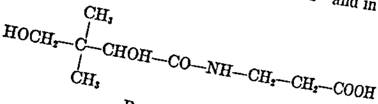
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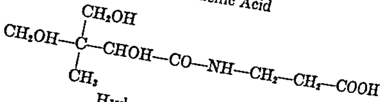
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soluble in chloroform and benzene.[4b] The substance is op
active, $[\alpha]_D^{25} = +37.5°$. Calcium pantothenate is also op
active, $[\alpha]_D^{26} = +24.3°$. For general purposes, calciu
pantothenate is the most desirable preparation. It occurs
fine, white, odorless, crystalline powder, possessing a slig
bitter taste. The compound is stable to light and air under
dinary conditions and is readily soluble in water. The aqueo
solutions, being alkaline, pH 8.5, are decomposed by ordinar
autoclaving. If sterile solutions are required, a Seitz or Berkefel
filter should be used. Other compounds, similar in structure have
been prepared but the biological activity is very low[18] and only one,
the hydroxyl compound prepared by Mitchell et al.,[19] shows as
much as 25 per cent of the activity of pantothenic acid. Failure
to obtain biologically active compounds by replacing the beta-
alanine portion of the molecule with other amino-acids,[19,20]
(beta-amino-butyric acid, aspartic acid, *l*-leucine) is indicative of
the structural specificity of pantothenic acid. Even the levo-
rotatory isomer is inactive when tested on rats[4b] and in microbio-
logical[15] assays.



Pantothenic Acid



Hydroxypantothenic Acid

Although often referred to as the chick dermatitis factor, it is
also necessary, in addition to maintaining a normal skin, for nor-
mal growth and normal development of the central nervous
system. In 1939 Jukes,[21] Woolley, Waisman and Elvehjem[22]
pointed out that the properties thus far described for "filtrate
actor" and the "chick dermatitis factor" were very similar to the
roperties of pantothenic acid—described by Williams et al.[2] in

1933. On a pantothenic acid-deficient diet the chick exhibits a very typical and specific dermatosis. Incrustations appear around the eyes, the corners of the mouth and between the toes.[1a,1b] There is a marked keratinization of the skin with subsequent sloughing. Feathering is retarded and the feathers appear very rough. Lesions occur in the central nervous system (degeneration of the myelinated fibers) and often fatty infiltration of the liver.[23] Snell et al.[24] found that the tissues of pantothenic acid-deficient chicks contained much less of the substance than normal animals. In certain tissues, brain, muscle, kidney, liver, the reduction in tissue concentration amounted to 50 to 65 per cent.

In the rat and in the mouse it has been demonstrated that a diet deficient in pantothenic acid causes a number of pathological changes; poor growth,[25,26] dermatitis, adrenal hemorrhages (not found in mice[25]) and a graying of the hair.[27a,27b,27c,27d] The post-mortem examination of rats that have been fed diets deficient in "filtrate factor" (pantothenic acid) showed extensive damage to the kidneys, necrosis and numerous hemorrhages being commonly observed.[28a,28b] The administration of pantothenic acid to the deficient animals caused not only an arrest of the degenerative changes but complete repair of the damages. Mills et al.[29] and Salmon and Engel[30] also described the curative effects of pantothenic acid on adrenal necrosis. Bowles et al.[31] observed that rats on a diet deficient in pantothenic acid or pyridoxine showed marked vascularization, thickening and opacity of the cornea. The data of Nelson et al.[32] indicate that the sparing action of a high protein diet on the pantothenic acid requirement of the rat cannot be explained by increased intestinal synthesis (measured by fecal excretion) or by a decrease in vitamin loss (by urinary excretion).

McCall et al.[33] reported that pantothenic acid deficiency in the monkey produced the following syndrome: retarded growth, diarrhea, cachexia, ataxia, anemia and thinning and graying of the fur. According to Wintrobe et al.[34] a deficiency of pantothenic acid in the young pig is characterized by: marked retardation of growth, diarrhea, loss of appetite, loss of hair, excessive nasal secretion and coughing, and ataxia. There is extensive colitis; the bowel is hypertonic, edematous, and hemorrhages are common.

It requires 500 $\mu g.$, or more, per kilo body weight to bring about correction of the gastro-intestinal symptoms, to cause gains in weight, restoration of normal blood values and growth of hair. The ataxia improves but there may not be complete restoration of function (there are definite histologic changes in the central nervous system). Fouts et al.[35] found that dogs deprived of factor II (chick antidermatitis factor) developed a syndrome characterized by loss of appetite, diarrhea (often bloody), marked loss of weight and moderate anemia (normocytic). Autopsy revealed fatty livers. Morgan and Sims[36] observed that pantothenate deficiency caused retarded growth, loss of appetite, bloody diarrhea and a marked graying of the hair in dogs and foxes. Scudi and Hamlin[37] found lower values for blood cholesterol, cholesterol esters, and total lipids in the blood of pantothenic acid-deficient dogs. They also found extremely fatty livers. Supplee et al.[38] postulated that pantothenic acid is necessary to maintain the integrity of the adrenal cortex since a deficiency of the vitamin results in macroscopic and histologic lesions of the cortical layer. In summary the authors state: "The data as a whole indicate the specificity of pantothenic acid for maintaining a normal balance of adrenal functions."

Hegsted and Riggs[39] found that chicks, on a purified diet, require about 900 $\mu g.$ per 100 gm. of ration for maximum growth and according to Gillis et al.[40] 650 to 800 $\mu g.$ of pantothenic acid per 100 gm. of ration was adequate for optimal hatchability and growth. Very little is known concerning the metabolism of pantothenic acid in man (or animals). Since the blood level remains constant and since all tissues contain some of the vitamin it is evident that the vitamin present in the foods of man and animal is absorbed. It is assumed that it is absorbed as an intact molecule and we must also assume that enzymatic hydrolysis frees the protein-bound material. Stanbery et al.[41] found in the presence of recognized human deficiencies of thiamin, riboflavin or niacin, there was also a marked reduction, as much as 50 per cent, in the blood concentration of pantothenic acid. By relatively large injections of the vitamin, Spies et al.[42] found a rapid increase in serum values, followed by a prompt (within 24 hours) return to normal.

The most active analog of pantothenic acid to be studied is pantothenyl alcohol (alpha-gamma-dihydroxy-N-(3-hydroxy-propyl) beta, beta-dimethylbutyramide). Pfaltz[43] found that pantothenyl alcohol was about as active as calcium *d*-pantothenate in the treatment of achromotrichia in black rats, alopecia in mice and upon the growth rate of the young of both rats and mice. From a study of the urinary excretion of pantothenic acid, Burlet[44] found that both man and the rat possess the ability to convert the alcohol to the acid. Rubin et al.[45] employing the physiological availability test of Melnick et al.[46] compared the availability of pantothenic acid and pantothenyl alcohol to normal man. The studies on seven normal male adults indicated that when taken together with food (at meal time) the pantothenyl alcohol showed superior availability. "When given in the postabsorptive state there was very little difference in the availability of the two compounds."

Feaster et al.[47] found that the heat processing of meat products caused 30 to 40 per cent loss of the pantothenic acid originally present but that the pantothenic acid values did not drop during storage for one year. Waisman et al.[48] determined the pantothenic acid content of a large variety of meats and found that 30 to 40 per cent of the vitamin was lost during processing and/or cooking. Rice and Robinson[49] reported that dehydrated pork retained 73 per cent and dehydrated beef retained 68 per cent of the original pantothenic acid content. Canned meats showed little or no loss of niacin, riboflavin or pantothenic acid when stored at 20 to 99°F. for 10 months. Cover et al.[50] found that stewed lamb and beef retained an average of 75 per cent of the pantothenic acid originally present, the vitamin being distributed quite evenly between solids (50 per cent to 60 per cent) and broth (40 to 50 per cent). When pressure cooking was compared with open kettle stewing there was only about 10 per cent difference in favor of the pressure cooked material. Cheldelin et al.[51] studied the loss of several B vitamins during cooking and found only a slight loss of pantothenic acid in vegetables, eggs and meats.

There are no specific chemical tests for pantothenic acid and, although the chick test of Jukes and Lepkovsky[7a,7b,52] has been used, most investigators now employ one of the microbiological

assays[53,54,55,56] proposed for the estimation of the vitamin activity.

Pantothenic acid is present in all animal tissues with the liver and kidney containing the highest concentration of the vitamin. The heart, brain, pancreas and lung contain much more pantothenic acid than the muscle tissues. The vitamin is produced in green plants after the photosynthetic properties are developed and is also produced by many microorganisms. In any tissue only a small amount exists in the free state, most of the pantothenic acid being bound to various proteins. The studies of Waisman, et al.,[43] Cheldelin and Williams[57] furnish excellent data on the pantothenic acid content of a large number of foodstuffs. The livers of our edible animals occupy first place on the food list with 4000 µg. or more per 100 gms. Beef brain, beef pancreas, beef and pork kidneys, eggs, oatmeal, and peanuts are also rich sources of the vitamin. From the total list of foodstuffs and from an actural consumption standpoint, Cheldelin and Williams[57] regard milk, eggs, potatoes and white bread as the important dietary sources of pantothenic acid for man.

There are no data to indicate human requirements for pantothenic acid. However, since it has shown that the rat, the mouse, the chicken, the dog, the fox, the monkey and the pig require pantothenic acid for growth, for maintenance of normal skin, normal pigmentation of the hair, and for protection against the characteristic and severe adrenal syndrome, it seems reasonable to assume that the vitamin is essential to man. It is apparent that bacterial synthesis in the intestinal tract plus the vitamin contained in the average diet provide amounts adequate for growth and maintenance of normal tissue functions.

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Chapter 10

Folic Acid (Pteroylglutamic Acid)

THE ABILITY to produce cataracts in rodents[1a,1b,2] by feeding a diet deficient in vitamin G caused Day et al.[3] to attempt similar experiments with monkeys. However, before advanced corneal changes developed, the monkeys died of fulminating changes in the blood elements (anemia, leukopenia, thrombocytopenia) accompanied by severe gingivitis, diarrhea and loss of weight. The survival time on the G-deficient diet averaged 56 days but one control animal fed the deficient diet supplemented with yeast lived more than 200 days without showing any of the above symptoms. These investigators did not observe any similarity of the monkey syndrome with that of canine blacktongue or human pellagra and suggested that yeast contained an unidentified member of the B complex. In subsequent papers[4a,4b] it was shown that the deficiency syndrome in monkeys could be prevented by yeast and certain liver fractions. Although present in the Cohn fraction G, commercial parenteral liver extracts used in the treatment and control of pernicious anemia were not effective sources of the new factor, now named "vitamin M." While developing a suitable diet for the riboflavin assay with chicks, Stokstad and Manning[5] observed that yeast and middlings exhibited growth-promoting effects which could not be explained by the filtrate factor and riboflavin content of the supplements (yeast or middlings). Since the new growth factor seemed to be unlike any of the known nutritional factors, Stokstad and Manning proposed that it be tentatively designated "Factor U." A series of reports by Bauernfeind and co-workers[6a,6b,6c,6d,6e] indicated that chicks and hens required unrecognized factor(s) for normal growth and reproduction. Since the active material was precipitated from aqueous extracts of yeast or cereals it became known as the "Alcohol Precipitate Factor." It soon became evident that the

material under investigation contained at least two factors, "R" and "S," and that they were not identical with any of the known nutritional factors required by the chick. It was noted that the only nutritional factor resembling factors R and S was factor U, recently reported by Stokstad and Manning.[5] Factor R was soluble in acid alcohol, while factor S was insoluble. Hogan and Parrott[7] produced an anemia in chicks by dietary means, and observed that the antianemia agent was not identical with any of the known vitamins. Because the new factor was apparently a member of the B complex and since it was essential for the chick it was designated "vitamin B_c." Snell and Peterson[8a,8b] observed hat *Lactobacillus casei* would not grow on a hydrolysed casein medium unless yeast or liver extracts were added. Using norite adsorption (in acid solution) the active material could be separated into two fractions. The fraction readily absorbed on norite produced growth effects on *L. casei* not duplicated by mixtures of any established growth factors. Mitchell, Snell and Williams[9] extracted four tons of spinach and subjected the concentrate obtained to a series of steps which finally yielded an acid nutrient in nearly pure form. The substance, or one with similar chemical and physiological properties was found to be present in relatively large amounts in liver and kidney tissue. It also was found to be widespread in Nature, grass, yeast, mushrooms and many green leaves being good sources of the substance.

The name "folic acid" (Latin folium) was suggested. The concentrated material stimulated the growth of *L. delbruckii* and *L. casei* under similar conditions and dosage.[8a 8b] Since 50 μg. of the concentrated nutrient per day produced a considerable gain in weight (over those on the control diet) Mitchell et al.[9] suggested that folic acid possessed vitamin properties. Tissue assays of the experimental animals indicated bacterial synthesis within the intestinal tract. Keresztesy et al.[10] compared the amount of folic acid and norite eluate factor in various substances, including liver extract and found some were less active for *L. casei* than for *Strep. lactis R.* A spinach concentrate was equally active for both organisms. These investigators felt that their experiments indicated the presence of a new growth factor—not identical with the norite eluate factor or folic acid. Hutch-

ings et al.[11a,11b] purified the norite eluate factor required by *L. casei* and demonstrated that it was an active growth factor for the chick. Mills et al.[12] had already shown that the Peterson eluate factor (from liver) acted as a growth factor for chicks and promoted normal feathering and formation of hemoglobin. Mills et al. also stated that their material was similar to Hogan's[7] antianemia factor, "Factor U"; and the "alcohol precipitate factor."[6a,6b,6c,6d,6e] Briggs et al.[13a,13b] felt that their work clearly indicated the presence of two new factors of the B complex required by the chick; one substance promoted growth the other promoted normal feathering. Vitamin B_{10} was proposed for the factor concerned with feathering and vitamin B_{11} for the factor concerned with growth. In April, 1943, Pfiffner et al.[14] reported the isolation of vitamin B_c in crystalline form and found that when the deficient diet was supplemented with B_c, 250 $\mu g.$ per 100 gm. of ration, chicks grew normally and did not develop an anemia. Stokstad[15] obtained crystalline fractions (methyl esters) of the "norite eluate factor" from both liver and yeast. The hydrolysed esters were found to be equally potent for *L. casei* but when tested with *S. lactis R*, the yeast preparation was only half as active as that from liver. Stokstad suggested that his material, isolated from liver, was probably identical with vitamin B_c isolated by Pfiffner et al. but that his material from both liver and yeast differed from the growth factor for *S. lactis R* reported by Keresztesy et al[10]. In 1944 Hutchings et al.[16] announced the isolation of a new *Lactobacillus casei* factor, the new compound *crystallized as the barium salt, the methyl ester* and the free acid, was very active when assayed with *L. casei* but only slightly active when tested with *S. lactis R*. On the basis of a comparison of absorption spectra, Hutchings et al. felt that the three *L. casei* factors thus far studied were different from folic acid.

Daft and Sebrell[17] produced granulocytopenia and leukopenia in rats (30 to 122 days on a diet containing one per cent of either sulfaguanidine or sulfasuxadine) and showed that three preparations of folic acid (all from crystalline material) were active in correcting the leukopenia and granulocytopenia. Campbell et al.[18] showed that the addition of as little as 20 $\mu g.$ per 100 Gm. of ration gave some growth protection but for complete treatment of

Year	Name	Activity
1935–38	Vitamin M	Cures nutritional cytopenia in monkeys
1938	Factor U	Growth factor for chicks
1938–40	Factors R and S	Required in chick nutrition
1940	Vitamin B_c	Cures chick anemia
1940–41	Norite Eluate Factor	Growth factor for *L. casei* and *S. lactis R*
1941	Folic Acid	Growth factor for *L. casei* and *S. lactis R*
1943	S. lactis R Factor	Active for *S. lactis R*, inactive for *L. casei*
1944	Crystalline L. Casei Factor (Fermentation)	Active for *L. casei*, inactive for *S. lactis R*
1943	Crystalline Vitamin B_c from (Yeast or Liver)	Active for chick, *L. casei*, and *S. lactis R*
1943–44	Vitamins B_{10} and B_{11}	Chick active, not active for bacteria
1944–45	Crystalline vitamin B_c Conjugate	Chick active, only slightly active for *L. casei* or *S. lactis R*
1945	Synthetic L. Casei Factor	Active for chick, monkey, rat and bacteria

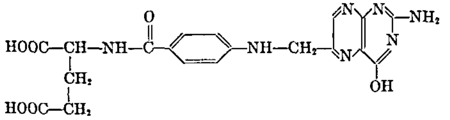
contained one mol of glutamic acid in peptide linkage with the carboxyl group of para-aminobenzoic acid. Complete degradation studies showed that both factors contained the same pteridines and both yielded para-aminobenzoic acid and both contained glutamic acid. The only difference being the one mole of glutamic acid in the liver factor. From the facts derived in these studies the structure for the liver *L. casei* factor was elaborated and the name, "Pteroylglutamic Acid" was given to the substance. From the degradation studies and from the synthesis of certain degradation products, Mowat et al.[25] established the molecular structure of the liver *L. casei* factor, N[4{[(2-amino-4-hydoxy-6-pteridyl) methyl]amino}benzoyl]glutamic acid. The total synthesis of the liver *L. casei* factor (pteroylglutamic acid) was announced in 1946 by Angier et al.[26a, 26b]

Harned et al.[27] studied the acute and chronic toxicity of the synthetic material with mice, rats, guinea pigs, rabbits, cats and dogs and stated: "...The action of this substance is characterized by the lack of response to the usual pharmacological tests. It has a low acute and chronic toxicity and an almost complete absence

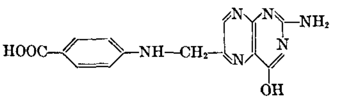
of side reactions, even when the dose is far above the therapeutic range."

Folic acid (pteroylglutamic acid) of commerce is yellow, crystalline powder, insoluble in water, alcohol, chloroform, ether or benzene. It is sparingly soluble in hot water or 0.1 *N* alkali.

Prior to the synthesis of folic acid it had been impossible to provide material of a high degree of purity for extensive clinical trial. In 1946–47 there appeared a large number of reports on



N[4{[(2-amino-4-hydroxy-6-pteridyl)methyl]amino}benzoyl]glutamic acid
Folic Acid (Pteroylglutamic Acid)



4{[(2-amino-4-hydroxy-6-pteridyl)methyl]amino}benzoic acid
Pteroic Acid

the use of folic acid in the treatment of the megaloblastic anemias, with most of the emphasis on the treatment of pernicious anemia, sprue and nutritional macrocytic anemia.[21,22a,22b,23] The early clinical reports include the studies of Spies et al.,[22a,22b,23] Moore et al.,[23] Darby and Jones,[21] Vilter et al.,[29] Hall and Watkins,[30] Meyer,[31] Suárez,[32] Goldsmith,[33] Heinle and Welch,[34] and Zuelzer et al.[35] It was established that the vitamin was as effective by

mouth as when given parenterally and this finding, together with the fact that none of the commercial parenteral liver extracts contained appreciable amounts of folic acid[36,37] (*L. casei* factor), indicated that the active material present in parenteral liver extract and folic acid were entirely different substances. Although the subject of much speculation, the relationship between liver extract and folic acid yet remains to be determined.

Before many months had elapsed it became evident that all aspects of pernicious anemia were not adequately treated or controlled by folic acid. The clinical data also indicated that folic acid therapy frequently failed to effect a complete hematologic remission[30,31] and that for several weeks the rate of erythrocyte regeneration and hemoglobin formation were below the standards established for parenteral liver extract. Hall and Watkins[30] treated 14 patients with pernicious anemia (in nine of these the diagnosis was made prior to treatment with folic acid) and observed that only three of this group had a hematologic remission comparable to that usually obtained with liver extract and four patients did not reach normal red cell levels after 10 weeks of folic acid therapy. Although it was observed that folic acid rapidly changed erythropoiesis in the bone marrow from a megaloblastic to a normoblastic type, three of the patients exhibited definite signs of peripheral nerve changes and combined system disease. Sprue, the nutritional macrocytic anemia and other megaloblastic anemias were better treated and controlled by folic acid therapy than by various types of liver therapy. During a period of nearly two years Zuelzer[35] studied blood and bone marrow of 15 infants (age five weeks to one year) and found that the administration of folic intramuscularly or perorally resulted in a prompt return to the normoblastic type of erythropoiesis. Spies et al.[29] treated nine cases of sprue with synthetic folic acid with rapid improvement of the diarrhea, the anemia and the glossitis. As a consequence of better absorption of nutrients, all patients gained weight. Suárez et al.[32] used synthetic folic acid in the treatment of 50 cases of sprue and found that 10 to 20 mg. per day was adequate for the treatment of all manifestations of the disease and that 2.5 to 5 mg. per day was an adequate maintenance dose for most patients.

Several clinics reported that some patients developed central nervous system changes and some patients, showing central nervous system changes before folic acid therapy was begun, showed progression. In early studies by Meyer[31] it was noted that three patients, out of a group of 12, reached stationary levels of red blood cells and hemoglobin and that there was a progression of signs of subacute combined sclerosis notwithstanding the daily administration of 20 to 50 mg. of folic acid. In an attempt to determine whether synthetic folic acid would control all of the signs and symptoms of pernicious anemia and sprue, Vilter et al.[29] observed the course of 28 patients, for one year. Although the hematologic remission was satisfactory in most instances four patients (free of neurologic involvement at the time of diagnosis) developed definite indications of combined system disease and a fifth patient showed progress of changes initially present. Heinle and Welch[34] reported an "explosive" rapidly progressive neurologic relapse in one pernicious anemia patient while being maintained on folic acid and also stated that folic acid not only fails to prevent but may actually precipitate combined system disease.

Wagley[38] reported that neurologic disturbances had reoccurred or developed in eight out of 10 patients (pernicious anemia) treated with folic acid and that the changes in the status of the patient were frequently abrupt and severe. Wagley[38] also noted that no neurologic disturbances were observed in sprue patients treated with folic acid. Ross et al.[39] treated 32 pernicious anemia patients with synthetic folic acid with satisfactory hematologic remissions but observed that the glossitis was often not completely relieved and eventually there were definite, gradual declines in blood levels (erythrocytes and hemoglobin). In this series of 22 patients, seven developed subacute combined system disease and in four others there was marked progression of the neurologic involvement. In reviewing the effects of folic acid in pernicious anemia an editorial[40] entitled *A Warning Regarding the Use of Folic Acid*, closed with the following remarks: "consequently the use of folic acid as a therapeutic agent appears to offer no benefit, but only risk to the patient. "The conflicting views as to the "toxicity" of folic acid, suggested the necessity of

carrying out extensive, long-term studies and late in 1947 three groups, each working independently, began to assemble patients. Each of two groups contained approximately 25 normal persons (mostly hospital technicians and attendants) and 25 persons with any form of anemia other than pernicious anemia; the third group was made up of 70 normal persons. Each of the 160 patients received 20 mg. of folic acid daily perorally for approximately one year. The neurologic status of each patient was determined prior to the inauguration of folic acid therapy and at monthly intervals during the observation period. The first of the three reports, that of Poliakoff et al.,[41] summed up their study as follows": ". . .This long-term study, constituting 768 patient-months on 20 mg. daily dosage of folic acid showed no neurologic toxicity" and ". .Signs related to subacute combined sclerosis or peripheral neuritis did not develop in any of the group." The second group of patients, studied by Weissberg et al.,[42] included 26 normal individuals and 22 patients with some form of anemia other than pernicious anemia. This careful study showed that folic acid given in 20 mg. doses daily for approximately one year had neither an adverse effect upon the central nervous system of normal persons nor any discernible effect upon the central nervous system of patients with anemia other than pernicious anemia. Harvey, Howard and Murphy[43] studied a group of 40 patients (36 women, 4 men) for varying periods of time up to one year, and although several of the women had a hypochromic anemia, failed to find any evidence of spinal cord or peripheral nerve damage during the course of study.

Although it has been reported that an occasional sprue patient did not respond favorably to folic acid therapy, Suárez[44] raises the question of diagnosis and states that in a series of 100 cases of sprue, many of whom had been treated for a year, he had never observed the development of neurologic changes following folic acid therapy. In reviewing his experience with sprue, covering a period of many years, Suárez stated that while the hematologic response to folic acid was equal to that obtained by the use of parenteral liver extract, the improvement in the gastro-intestinal symptoms was more striking than that observed from liver therapy. In conclusion Suárez states: "We have yet to see, in

this part of the world, any harmful effect that could be attribute to the administration of pteroylglutamic acid."

The implication of Ross et al.[39] and also the editorial,[40] th pteroylglutamic acid might function as a metabolic antagoni for glutamic acid led Franklin et al.[45] to study the utilization glutamic acid, in the presence of varying amounts of folic aci by bacteria. Increasing the folic acid content of the medium b 1000 times had no effect upon the growth of *L. casei* or *S. faecalis* Likewise the respiration of rat brain slices was not inhibited b large amounts of folic acid, nor was the accelerated respiratic caused by adding glutamic acid to the medium retarded by hig concentrations of folic acid.

Recent comments[46] on this subject (toxicity) indicate that th evidence accumulated during 1948–49 has been sufficient to di prove the bogey created in 1947. The following brief statemen adequately sums up the voluminous discussion on this que tion ". . .that folic acid does not offer neurologic protection pernicious anemia patients is clear, but that it actually does har to nervous-system tissue directly appears to have been di approved."

Welch and co-workers[47] found that patients with perniciou anemia in relapse could not utilize the pteroylheptaglutamic aci (yeast) either before or after incubation with normal gastr juice (orally) or when the heptaglutamate was given intramu cularly. There was also almost no excretion of the free pteroylgl tamic acid (and no conjugate) in the urine of these perniciou anemia patients. After two successive intramuscular injectior of 2.8 mg. of the heptaglutamate a normal person excreted 8 pe cent as free acid but no conjugate. Bethell et al.[48] showed th pernicious anemia patients vary considerably in the ability utilize the natural occurring conjugates (yeast) of folic acid.

Weir and Comfort[49] treated seven cases of nontropical spr with synthetic folic acid and were surprised to find that thes patients showed little or no improvement. The diarrhea an steatorrhea present prior to therapy persisted all through th period of treatment with folic acid and as a result there was n gain in weight. Davidson et al.[50] had also found folic acid ine fective in idiopathic steatorrhea and celiac disease.

Denko et al.[51] reported that young men on a normal diet excreted 4 to 6 μg. of pteroylglutamic acid per day. Suárez[52] reported similar figures and stated that the excretion of pteroylglutamic acid after oral or parenteral administration, varied with the dose given. On a dose of 1 mg. (intramuscular administration) one patient excreted 16 per cent within 24 hours; with 10 mg. dose (either oral or parenteral administration) the excretion varied from 35 to 50 per cent. Suárez[52] also showed, by measuring urinary excretion of pteroylglutamic acid, that sprue patients possessed the ability to hydrolyze and utilize the pteroyltriglutamate. Steinkamp et al.[53] gave varying doses (5 to 16 mg.) of pteroylglutamic acid to each of nine normal persons and found that the group excreted an average of 28 per cent of the administered dose. Each of nine hospitalized patients receiving the same doses as above showed a much lower urinary excretion. Swendseid et al.[54] *found that six normal subjects excreted about 3 μg.* per day during the control period and after administration of 4 mg. excreted approximately 3.2 per cent within 24 hours. Following the administration of doses from 1 to 15 mg. of pteroylglutamic acid to each of 8 normal adults, Jukes et al.[55] found the six hour urinary excretion varied from 20 to 77 per cent, with an average of about 44 per cent. When 7.9 mg. of pteroyltriglutamic acid (equivalent to 5 mg. of pteroylglutamic acid) was given the six hour excretion of pteroylglutamic acid averaged about 49 per cent and when 6.5 mg. of pteroyldiglutamic acid (equivalent to 5 mg. of pteroylglutamic acid) was given, the six hour urinary excretion averaged about 27 per cent.

Wilson et al.[56] produced a nutritional deficiency syndrome in monkeys (neutropenia, lymphopenia, anemia) and effected clinical and hematologic remissions by the administration of a yeast autolysate (*L. casei* factor) or purified *L. casei* factor from liver. The work demonstrated that the *L. casei* factor (folic acid) was essential for normal hematopoiesis and nutrition in the monkey. By the use of a folic acid antagonist (crude methyl folic acid) Cartwright et al.[57] produced in swine the hematologic manifestations of folic acid deficiency. Increasing the protein (casein) content of the diet had no significant effect on the deficiency syndrome, likewise the injection of large doses of parenteral

liver extract (as much as 150 units) did not appreciably alter the blood picture. In three instances the administration of folic acid led to the restoration of the bone marrow to normal and in four cases the anemia was promptly and completely relieved by folic acid therapy. Spicer et al.[58] produced agranulocytosis, leukopenia and hypocellularity in rats by adding a sulfa compound to a purified diet. The dyscrasias were prevented or successfully treated by certain liver extracts. Subsequently Daft and Sebrell[17] successfully treated this syndrome with crystalline folic acid.

Using *S. faecalis* (*S. lactis R*) as the test organisms Schweigert and Pearson[59] determined the free and protein-bound folic acid in the whole blood and plasma of man, the pig, the horse, cattle, the chicken and the turkey. There is a wide variation in the amount of this vitamin present in the whole blood and plasma of various animal species. The average values obtained by Schweigert and Pearson expressed in μg. per ml. are as follows:

Specie	*Whole Blood*	*Plasma*
Man	0 85	less than 0.5
Pig	6 6	6.1
Horse	3 3	3 5
Cattle	1.9	less than 0 5
Chicken	8.7	3.1
Turkey	16 8	6.0

Treatment of whole blood or plasma with an enzyme (takadiastase) resulted in a marked increase of folic acid in the blood and plasma of four species, varying from 20 μg. per ml. (pig, cattle) to 40 μg. per ml. (man, chicken). The work showed that the protein-bound vitamin was not available to *S. faecalis* for growth until freed by enzymatic treatment.

Hutchings et al.[60] prepared pteroylospartic acid and found that the substance inhibited the utilization of folic (pteroylglutamic) acid for growth for the chick, for *L. casei* and for *S. faecalis R.* The aspartic compound did not interfere with the utilization of folic acid by the rat. Sarett[61] has recently shown the growth of *L. arabinosus*, in the presence of an excess of para-aminobenzoic acid is accompanied by the production of pteroylglutamic acid with maximum concentration reached in about 20 hours. An

excess of para-aminobenzoic acid had no effect upon the utilization of pteroylglutamic acid by *L. casei*. Edwards et al.[62] and Woolley and Pringle[63] have prepared a number of compounds which inhibit the growth of certain bacteria competitively with folic acid.

With the enthusiasm which usually prevails when some use of a new therapeutic agent becomes established, it is not surprising that most investigators forgot that folic acid was a member of the B complex. Aside from the bone marrow studies and those dealing with excretion no attempts were made to determine the effects, if any, of folic acid upon general metabolic activities. The recent report by Grubbs et al.[64] marks an important step in this direction. As experimental subjects Grubbs et al. used seven normal adults (two women, five men), and 6 clinical patients; three with pernicious anemia, one with cirrhosis, one with Hodgkin's disease and one case of idiopathic steatorrhea. From their investigations the authors were able to point out five definite metabolic effects produced by the adminsitration of folic acid:

(1) A marked increase in appetite, accompanied by a definite euphoria.
(2) The basal oxygen consumption of the three pernicious anemia patients was definitely reduced after folic acid had been given.
(3) Five of the controls and all of the patients with macrocytic anemia showed an increased utilization of carbohydrate. This effect was most noticeable in the patients having the best hematopoietic response to folic acid.
(4) In all clinical subjects and in some of the controls there was an increased specific dynamic action during the period of folic acid therapy, the authors pointed out that this effect may be due to better absorption from the intestinal tract.
(5) [illegible] subjects there [illegible] d fecal excre- [illegible] ary excretion in three of the five subjects. The normal subjects showed a marked decrease (as much as 20 per cent) in nitrogen retention.

Using *S. faecalis* and *L. casei* as test organisms and employing water extraction, acid and alkali hydrolysis, as well as enzymatic treatment (takadiastase), Olson et al.[65] determined the folic acid content of a large number of foodstuffs. Enzymatic treatment gave the highest values by both test organisms. The richest

dietary sources for man were mammalian liver and the fresh, deep green leafy vegetables. In general, fresh green vegetables are good dietary sources of the vitamin. Because of general use breakfast cereals prepared from wheat are also important dietary sources. Storage of vegetables at room temperature results in large losses of folic acid. Ives et al.[66] determined the pyridoxine, biotin and folic acid content of over 100 samples of canned foods (mostly vegetables). As determined by assays with *S. lactis* and *L. casei* the solid contents of various canned products retained about 65 per cent of the folic acid. Schweigert et al.[67] determined (using *S. lactis* and *L. casei*) the folic acid content of various portions of meat (veal, beef, pork, lamb). Liver and kidney contain much more folic acid than the muscle portions. Cooking of various cuts of meat resulted in heavy losses (54 to 92 per cent) of folic acid. Cured pork hams retained about 65 per cent of the original folic acid content.

Folic Acid is a relatively new addition to the list of B-complex factors and it is not possible to state the requirements of man and various animal species for this vitamin.

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Chapter 11

Vitamin B_{12} and B_{12b}

(Cyano cobalamin and Cobalamin)

WHILE searching for a microorganism that required a rat growth factor present in refined liver extracts, Shorb[1] noted that *Lactobacillus lactis* Dorner required two unidentified growth factors (using amino acid basal medium plus all of the synthetic B vitamins). One factor was found in tomato juice, casein, and many other substances; the second factor, heat-stable, was found in relatively high concentrations in the refined, concentrated liver extracts used in the treatment of pernicious anemia. In brief, the *Lactobacillus lactis* Dorner (L.L.D.) factor was found to be concentrated in liver extract in almost a linear relationship to the effectiveness in the treatment of pernicious anemia. On the basis of this work, Shorb suggested that L.L.D. factor might be the substance effective in the treatment of pernicious anemia.

In April, 1948, just about 23 years after Minot and Murphy[2] had fed whole liver to the first group of pernicious anemia patients, Rickes et al.[3] announced the isolation of crystalline material, vitamin B_{12} (from liver extract) which, in microgram doses, produced marked hematologic responses in pernicious anemia in relapse. Rickes et al. found that the various commercial brands of parenteral extract varied widely in their content of the L.L.D. factor, from 13,000 to 154,000 L.L.D. "units," or from 1.3 to 14.0 μg. per cc. Microbiological assays of the crystalline B_{12} by Shorb[4] indicated from 11,000 to 17,000,000 L.L.D. units per milligram, depending upon test conditions, and demonstrated the unusual biological potency of the new compound.

West[5] obtained positive hematologic responses in each of three pernicious anemia patients by the intramuscular injection of 3, 6, and 150 μg. of B_{12} respectively. Spies et al.[6] gave 6 to 15

μg. of B_{12} to two patients with pernicious anemia, two patients with nutritional macrocytic anemia, and to one patient with nontropical sprue. Good hematologic and clinical responses were observed in pernicious anemia and definite hemopoietic activity was indicated in the nutritional anemia and in nontropical sprue. An early report of Spies et al.[7] showed that the parenteral injection of 8 μg. of B_{12} produced a definite clinical and hematologic response each of two patients with tropical sprue. A pernicious anemia patient who had suddenly developed acute combined system disease was treated with 5 μg. of crystalline B_{12} daily for 8 days, by Berk et al.,[8] with an excellent hematologic response. Marked improvement in the neurologic involvements was evident by the tenth day.

Independent and simultaneous studies of Smith[9a, 9b] resulted in the isolation of needle-shaped crystals of the antianemia factor from liver and several clinical tests with pernicious anemia patients in relapse verified the unusual biological activity. Smith was the first to detect the presence of cobalt in the crystalline material and examination of four different lots of crystals showed that the dried compound contained 4 per cent of cobalt. Rickes et al.[10] verified the presence of cobalt by emission spectrographic analysis and suggested that the reddish color of the crystalline material was associated with the presence of a cobalt complex. Both Rickes and Smith determined the presence of phosphorus. As tested by microbiological assay, autoclaving for 15 minutes at 121° C. does not impair the activity of vitamin B_{12}. The substance is rapidly inactivated by treatment with alkali (NaOH)-concentration of the alkali and time determine the rate of inactivation. Treatment with strong mineral acid (HCl) more slowly inactivates the vitamin.

Subsequently Ellis and co-workers,[11a, 11b] using a different procedure, obtained a red crystalline compound from Anahaemin (from liver) which appeared to be identical with the vitamin B_{12} isolated by Rickes et al.[3, 10] and Smith.[9a, 9b] After drying in vacuo, the Ellis compound contained 4 per cent of cobalt, identical with the figure reported by Smith. Aqueous solutions exhibited characteristic light absorption. In visible light a main band appeared at 500 $m\mu$ and a secondary band at about 520 $m\mu$;

While the molecular weight has not been definitely established, a consideration of known facts indicates a molecular weight of about 1500.

Kaczka et al.[16] treated vitamin B_{12} with hydrogen in the presence of a Pt catalyst and obtained a crystalline product which contained cobalt and phosphorus in about the same amount as vitamin B_{12}. The spectrum of an aqueous solution was different from that of B_{12} although the new substance processed marked biological activity it was much less active than B_{12} in the rat (growth response) and in pernicious anemia. The new material was designated vitamin B_{12a}. Brockman et al.[17] hydrogenated B_{12} (Pt catalyst in an atmosphere of hydrogen for 20 hours) and obtained crystalline material which showed an extinction coefficient characteristic of vitamin B_{12b}. The infrared absorption spectrum was also similar to that of B_{12b}. As measured by *L. leichmannii* and the chick, B_{12}, B_{12b} and the compound under study were equal in biological activity. The authors expressed the opinion that under the conditions of the experiments B_{12b} had been produced by the hydrogenation of vitamin B_{12}. Lang and Chow[18] reported that incubation of vitamin B_{12} with strong reducing agents (ascorbic acid, cysteine, hydroquinone) resulted in a marked loss of B_{12} activity (determined by spectrophotometric transmittance and microbiological assays).

Brink *et al.*[19] observed that hydrogen cyanide was liberated during the oxidation of B_{12} (in dilute sulfuric acid at 0°C. with potassium permanganate) and felt that the experimental observations indicated that vitamin B_{12} contains one cyano group bound coordinatively to the cobalt atom. The lack of toxicity[20] of vitamin B_{12} suggests that the cyano group is firmly bound in the complex. It is apparent that B_{12a} and B_{12b} are identical and do not possess the cyano group coordinatively bound in vitamin B_{12}. Solutions of vitamin B_{12a} yield vitamin B_{12} when treated with cyanide ions.[21] Since aqueous solutions of B_{12a} behave as a weak base (pH about 9) it is apparent that the cyano group has been replaced by a hydroxo group. Since there may be many modifications of the B_{12} molecule Kaczka *et al.*[21] suggest that the term *cobalamin* be used to designate the B_{12} molecule minus the cyano group. Following the proposed nomenclature B_{12} is designated

as *cyano-cobalamin* and B_{12a} becomes hydroxo-cobalamin. Other analogues (chloro-, sulfato-) have been prepared and converted to the cyano-cobalamin by treatment with cyanide ions.

In a search for sources of B_{12} other than liver, Rickes et al.[22] examined a wide variety of substances and found that powdered milk, beef extract and culture broths from several microorganisms showed marked activity for *L. lactis*. From the culture broth of *Streptomyces griseus* this group isolated a red, crystalline compound which by emission spectrographic analysis was shown to contain cobalt and phosphorus. The refractive index was also similar to that observed for the vitamin derived from liver. Erf and Wimer[23] compared the therapeutic effectiveness of vitamin B_{12} obtained from liver with that obtained from *Streptomyces griseus* and came to the conclusion that the two substances produced similar results and that the two materials were closely related or identical. Pierce et al.[24] obtained small, rod-like crystals from cultures of *Streptomyces aureofaciens* which were shown to contain cobalt and phosphorus. The compound showed absorption spectrum maxima at 273, 351 and 525 $m\mu$. The crystals were biologically active (chick and *L. leichmannii*). The authors suggested the term vitamin B_{12b} for the new compound. Stokstad et al.[25] isolated crystalline material from *Streptomyces aureofaciens* which exhibited different absorption spectra than B_{12}. The new compound, B_{12b}, and B_{12} showed equal biological activity for *L. leichmannii* and the chick. Fricke et al.[26] compared the chemical and physiological properties of B_{12} and B_{12b}. With *L. leichmannii* and with *E. coli* the two compounds showed equivalent activity and rats maintained on B_{12} deficient diets gave similar growth responses. If B_{1} [illegible] ere injected into a rat B_{12} was recovered in the urine, if B_{12b} [illegible] jected B_{12b} was subsequently found in the urine. Spies [illegible] ed B_{12b} in the treatment of four patients with pernicio[illegible] in relapse a[illegible] obtained satisfact[illegible] hemopoietic and c[illegible] es. The liminary clinical [illegible] tropical sprue a[illegible] al macro anemia suggest [illegible] n B_{12b} is [illegible] ese dis

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tologic response in pernicious anemia was good and improvement in neurologic involvement was noted. Jones et al. came to the conclusion that more than 1 μg. per day was necessary for the maximal rate of erythropoiesis. The results with the one case of nutritional macrocytic were about the same as those obtained by the use of liver extract but the response of the sprue patient was not as good as with either liver extract or folic acid. Hall and Campbell[29a, 29b] used vitamin B_{12} in the treatment of 11 patients with pernicious anemia and found that small doses (at the rate of 1–3 μg. per day) induced good hematologic responses and general clinical improvement including rapid disappearance of the glossitis.

Beard et al.[30] treated pernicious anemia patients with varying doses of vitamin B_{12} and came to the conclusion that one (1) μg. of crystalline B_{12} was the approximate equivalent of 1 U.S.P. Injectable Unit of refined liver extract. For initial therapy Beard et al. recommended the injection of 200 to 300 μg. followed by monthly injections of 30 μg. Bone marrow studies, done serially, showed that B_{12} therapy brought about a very rapid change from megaloblastic to normoblastic activity, the change being complete in 48 to 72 hours. Schieve and Rundles[31] found that the lingual manifestations of pernicious anemia (in relapse), which frequently respond poorly to folic acid therapy, respond rapidly and completely to the administration of vitamin B_{12}. Stone and Spies[32] reported that the severe, fiery red lesions of the mucous membranes, frequently observed in patients with pernicious anemia, benefited by liver therapy but usually not by folic acid therapy, were controlled by injections of vitamin B_{12}.

Mueller et al.[33] treated a number of pernicious anemia patients with vitamin B_{12} and obtained complete remissions in the bone marrow, the blood, the neurologic system and concluded that the vitamin was as effective as liver extract in the treatment of this disease. There was no evidence, from their work or that of others, that vitamin B_{12} was more effective than liver extract or that it would induce a remission, differing in any respect from that produced by liver extract. For the routine treatment of pernicious anemia in relapse the authors suggested the administration of 5 μg. daily for 10 days and 5 μg. per week thereafter. Severe

neurologic involvements are an indication for higher dosage, 10 μg. daily for several months.

Berk et al.[34] were the first to show that small peroral doses (5 μg.) of B_{12} produced no hematologic response in patients with pernicious anemia. Simultaneous administration of large amounts (125–150 cc.) of neutralized normal gastric juice with 5 μg. of B_{12} produced significant increases of reticulocytes and erythrocytes but did not equal the hematologic and clinical responses elicited by the intramuscular injection of 5 μg. of vitamin B_{12}. Hall and co-workers,[35] using Berkefeld-filtered, pooled, normal gastric juice confirmed the findings of Berk et al. and showed that the potentiation by gastric juice could be demonstrated when the vitamin was given 2 hours before the gastric juice. It was also found that the peroral administration of a single dose of 25 μg. daily for seven days failed to induce a hematopoietic response. The daily oral administration of 150 cc. of filtered pooled gastric juice was also without effect. However, 5 μg. of B_{12} plus 25 cc. of Berkefeld-filtered, pooled normal human gastric juice produced a submaximal hematopoietic response. Increasing the amount of gastric juice, up to 150 cc., produced the maximum hematopoietic response. These studies indicate that this recently discovered component of the B complex, B_{12}, and Castle's "extrinsic factor" are either closely related or identical substances. Bethell et al.[36] also showed that desiccated extracts of hog duodenal mucosa possessed no hemopoietic activity alone but when combined with 5 μg. of B_{12}, induced hematologic and clinical remissions in pernicious anemia patients in relapse.

Wolf et al.[37] demonstrated that normal human gastric juice contains a small amount of vitamin B_{12} (assays with *L. lactis*). Experimental work also indicated that neither the structure nor the microbiological activity was modified by the incubation of B_{12} and gastric juice at pH 2 or pH 7 (at 37°C.). The authors felt that these studies afforded strong support for the hypothesis that the primary action of the intrinsic factor depended upon its capacity to promote the absorption of vitamin B_{12} from the intestinal tract. The studies of Dyke et al.[38] lend additional strong support to the hypothesis that the intrinsic factor is con-

cerned with the absorption of vitamin B_{12} from the intestinal tract.

Dyke et al.[38] also demonstrated the presence of vitamin B_{12} in the contents of the alimentary tract at various levels in man and animals and showed that the highest concentration of the vitamin is found below the ileocecal valve. It seems apparent that in man the normal requirements of B_{12} may be provided through bacterial synthesis. Both Berk et al.[34] and Bethell et al.[39] found that untreated pernicious anemia patients excrete large amounts of vitamin B_{12} in the feces and suggest that excessive elimination indicated an inability on the part of such patients to absorb the vitamin present in the food ingested or that synthesized by intestinal bacteria.

According to Charkey et al.[40] chicks receiving B_{12} supplements grew more rapidly and utilized feed more effectively than the B_{12}-deficient controls. The authors suggest that B_{12} promotes the utilization (tissue building) of circulating amino acids. Couch et al.[41] compared the B_{12} content of tissues in two groups of birds: (1) chicks on a basal diet (35 per cent soybean oil meal); and (2) chicks receiving the same basal diet plus the B_{12} supplement, 20 μg. per kilo ration. At the end of 10 weeks the chicks receiving the B_{12} supplement averaged 280 grams more than those control groups. The livers of the B_{12} group contained about five times and the kidneys about two times as much B_{12} as the same organs from the control group.

Colby and Ensminger[42] carried on extensive experiments with young pigs and found that supplementing the basal diet with 30 μg. of B_{12} per kilo of ration in no way added to the growth response or to the general well being of the animals. Likewise the injection of 12 μg. of B_{12} per week was without effect. Quite different results were reported by Neumann et al.[43] who fed 48 hour-old pigs with an alpha-protein "synthetic milk" ration containing 2 grams of sulfasuxidine per liter of milk (sulfasuxidine was added to inhibit intestinal synthesis of B_{12}). The addition of B_{12} produced favorable growth responses and when the diet contained 50 μg. of B_{12} per kilo of dry ration optimal growth was obtained. Nesheim and Johnson[44] studied the B_{12} requirement of baby pigs (alpha-protein milk ration described by Neumann et al.[43]) and from the observations made on 29 young pigs came

neurologic involvements are an indication for higher dosage, 10 μg. daily for several months.

Berk et al.[34] were the first to show that small peroral doses (5 μg.) of B_{12} produced no hematologic response in patients with pernicious anemia. Simultaneous administration of large amounts (125–150 cc.) of neutralized normal gastric juice with 5 μg. of B_{12} produced significant increases of reticulocytes and erythrocytes but did not equal the hematologic and clinical responses elicited by the intramuscular injection of 5 μg. of vitamin B_{12}. Hall and co-workers,[35] using Berkefeld-filtered, pooled, normal gastric juice confirmed the findings of Berk et al. and showed that the potentiation by gastric juice could be demonstrated when the vitamin was given 2 hours before the gastric juice. It was also found that the peroral administration of a single dose of 25 μg. daily for seven days failed to induce a hematopoietic response. The daily oral administration of 150 cc. of filtered pooled gastric juice was also without effect. However, 5 μg. of B_{12} plus 25 cc. of Berkefeld-filtered, pooled normal human gastric juice produced a submaximal hematopoietic response. Increasing the amount of gastric juice, up to 150 cc., produced the maximum hematopoietic response. These studies indicate that this recently discovered component of the B complex, B_{12}, and Castle's "extrinsic factor" are either closely related or identical substances. Bethell et al.[36] also showed that desiccated extracts of hog duodenal mucosa possessed no hemopoietic activity alone but when combined with 5 μg. of B_{12}, induced hematologic and clinical remissions in pernicious anemia patients in relapse.

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llis and Norris[52] confirmed this interrelationship between tamin B_{12} and methylating compounds. Using a special eparation of yeast as the sole source of protein, György and ose[53] produced a severe hemorrhagic necrosis of the liver in rats dietary measures. The 13 rats, receiving 1 μg. of B_{12} daily by e mouth and the 12 rats used as controls (no B_{12}) died at ap- oximately the same time, 34 to 36 days, from typical, extensive morrhagic necrosis. In subsequent experiments, with rats on w protein-low fat diet it was found that 0.5 μg. of B_{12} daily rally) exerted significant lipotropic activity.

Using the rat assay, quite specific for vitamin B_{12}, Lewis et al.[54] termined the B_{12} activity of a number of natural products. he glandular meats (liver, kidney) are the richest food sources of $_{12}$. Muscle tissues of edible animals, eggs and milk contain uch less B_{12} but are important food sources because of frequent e in the diet. Plant tissues do not contain measurable amounts vitamin B_{12}.

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Wetzel *et al.*[46] found that the daily oral ingestion of 10 μg. of crystalline B_{12} produced substantial growth responses in 5 of 11 school children. These studies on malnourished children seemed to clearly indicate a functional deficiency which was benefited by the oral administration of B_{12}. Downing[47] studied a group of 48 premature infants and after administering 10 μg. of B_{12} intramuscularly at intervals of one to three days, came to the conclusion that B_{12} had no effect in promoting weight gain in premature infants.

Hall and Drill[48] found that profuse, progressive hepatic fibrosis produced in rats by high fat diet could be prevented by choline supplements or by the injection of crude liver extract. It was shown that the liver extract did not contain sufficient choline to account for the observed lipotropic action. Drill and McCormick[49] showed that the administration of a B_{12} concentrate to rats on a high fat diet exhibited a pronounced lipotropic effect, similar to the effects produced by the administration of liver extract. The concentrate supplying 1 μg. of B_{12} contained only 0.5 mg. of choline and 0.19 mg. of methionine, hence these agents could not explain the observed lipotropic effects.

Stokstad et al.[50] have presented experimental evidence which shows that vitamin B_{12} is involved in the methylation of homocystine to methionine. When the chick diet described contained B_{12}, both homocystine and methionine here observed growth response. In the absence of B_{12} only methionine showed a growth responses. Schaefer et al.[51] demonstrated that they could prevent the "hemorrhagic kidney" in rats and promote growth in rats by suboptimal amounts of choline and methionine, provided that B_{12} was added to the diet (3 μg. per 100 gm.). Using chicks,

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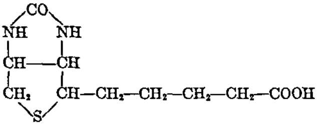
Chapter 12

d-Biotin (Vitamin H, Coenzyme R)

MORE THAN 50 years ago Wildiers discovered that yeasts would not grow on synthetic media unless some substance present in beer wort and in all growing cultures of yeast was provided. This growth promoting substance for yeast was called "bios" and subsequently the complex effects ascribed to bios resulted in the postulation of more than one bios factor (bios IB and bios IIB). In 1916 Bateman[1] found that the inclusion of large amounts of egg white in experimental diets produced toxic symptoms in the rats. A decade later Boas[2] found that rats maintained on a diet containing large amounts of raw egg white as the sole source of protein developed a number of characteristic deficiency symptoms (dermatitis, loss of hair and muscular incoordination). In addition to these symptoms, Lease et al.[3] and Findlay and Stern[4] observed neural disturbances in their experimental animals and applied the term "egg white injury" to the syndrome. Boas also discovered that yeast liver and certain other foods contained a substance, designated vitamin H by György,[5a,5b] which protected rats against the egg white injury. It was also observed that the symptoms did not develop when the egg white had been cooked. In 1936 Kögl and Tönnis[6] were successful in resolving the yeast growth factors and isolated a crystalline substance from egg yolk which they called "biotin" (from 250 kilos of dried egg yolk they obtained 1.1 mg. of crystalline biotin). Working along a somewhat different line Allison et al.[7] isolated a growth promoting substance for *Rhizobium* which they named "Coenzyme R." Later the studies of West and Wilson[8] with yeast and *Rhizobium* indicated that biotin and coenzyme R were identical in growth-promoting activity. In 1940 György et al.[9] announced that vitamin H, biotin and coenzyme R were different names for a single substance. Soon after du Vign-

eaud et al.[10a,10b] announced the empirical and structural formulae (1941–42) Harris et al.[11a,11b] described (1943–44) the details of the synthesis of *d*-biotin (the naturally occurring form).

Pure crystalline *d*-biotin (colorless needles) is sparingly soluble in water (30 to 40 mg. per 100 cc. at 25°) and alcohol (60 mg. in 100 cc. at 25°) and insoluble in ether, acetone or ethyl acetate. By neutralization of the acid carboxyl group with sodium carbonate, etc., it is possible to prepare solutions containing as much as 20 grams of the sodium salt per 100 cc. The pure vitamin melts at 231–232°C. (the methyl ester at 166–167°C.) and is characterized by a high degree of heat stability. Biotin is not light sensitive nor is it affected by ordinary reducing agents. It is inactivated by strong oxidizing agents (hydrogen peroxide, potassium permanganate) and by treatment with strong alkalies or acids. Biotin is optically active, $[\alpha]_D^{26} = +91°$ (2 per cent solutions in 0.1N NaOH).



Biotin

A number of investigators reported that the egg white injury syndrome in the rat included the typical denudation around the eyes,[12,13,14] "spectacle eye" condition, and that it could be prevented or cured by the administration of biotin.[15] The skin lesions observed in biotin-deficient rats are not primarily the peripheral type such as are seen in pellagra or the acrodynia of pyridoxine deficiency but are characterized by seborrhea and exfoliation. Lease and Parsons[16] showed that a diet rich in raw egg white caused a typical dermatitis in chicks, and the work of Eakin et al.[17a,17b] demonstrated that the deficiency disease, observed in rats and chicks, was caused by the "antivitamin," avidin, present in raw egg white. Crystalline avidin, isolated by György et al.[18] in 1941, was shown to inactivate

biotin. Biotin-deficient chicks develop a specific dermatitis[1] which includes the following symptoms: the bottom of the fee become rough, encrusted and in extreme cases develop hemorrhag cracks. Lesions also appear around the corners of the mout and the eyes become edematous and frequently closed by sticky exudate. Hegsted et al.[19b] showed that many foods co tained a factor capable of curing the chick dermatitis and note its similarity with vitamin H. About the same time Ansbach and Landy[20] produced biotin deficiency in chicks by feeding biotin-deficient diet (less than 0.1 μg. daily) and subsequent cured the symptoms by feeding 1.25 μg. daily. This wo avoided all use of avidin and established biotin as a member the ever-growing vitamin family. Ansbacher[21] produced peros in chicks by limiting the biotin intake and Jukes and Bird pr duced the same condition in a much shorter time by feeding diet which contained 30 cc. of raw egg white per 100 gram Jukes and Bird[22] found that the dermatitis and perosis were pr vented by the parenteral administration of 0.34 μg. of biotin p day. Richardson and co-workers[23] felt that it required two five μg. of biotin per day to prevent the development of de matitis in the chick. These investigators also reported th perosis developed in 8 to 14 days but the dermatitis did not b come evident until about twenty-eight days (raw egg whit sucrose ration). Cravens et al.[24] reported that hens maintain on a low biotin intake, but not low enough to cause dermatiti produced eggs of low-hatchability. Patrick et al.[25] found th turkey poults developed a dermatitis, similar to that seen chicks, which could be prevented or cured by substances co taining *d*-biotin. Waisman and associates[26] produced chron deficiency in monkeys and Wiese et al.[27] found that biotin pr vents or cures a posterior paralysis produced in calves fed a sy thetic diet.

Sydenstricker et al.[28] produced typical biotin deficiency symp toms in man in a few weeks by feeding large amounts of raw eg white (thirty per cent of daily caloric intake). The symptoms ob served included; characteristic exfoliative dermatitis, pronounce pallor, mental depression, and general lassitude. All of thes symptoms were rapidly cured (three to five days) by the adminis

tration of a biotin concentrate equivalent to 150 to 300 μg. daily. Biotin has been used with varying degrees of success in the treatment of acne vulgaris[29] and cases of severe seborrhea (psoriasis).

Biotin is widely distributed in both plant and animal tissues. Schweigert et al.[30] showed that liver and kidney are the richest sources of the vitamin. Heart muscle, chicken meat, and milk are also good sources. Ives et al.[31] determined the pyridoxine, folic acid and biotin content of 101 samples of various canned foods and reported that salmon, yellow corn, spinach and peas were excellent food sources of biotin. (2–10 μg. per 100 gms.). An average of about 75 per cent of the biotin remains in various meats after cooking. Cheldelin et al.[32] examined the losses of various vitamins in 30 commonly used foods and stated that biotin losses were irregular, more was lost in meats than in vegetables.

The rat assay procedures of Boas,[2] György,[5b] the chick assay suggested by Hegsted et al.[19a, 19b] and several microbiological methods (*Lactobacillus arabinosis*,[33] *Clostridium butylicum*[34]) have been proposed for the determination of the biotin in various foods and biological products. Although man, monkeys, dogs, rabbits, guinea pigs, rats, chickens, turkeys require biotin, it is certain that man, and probably many animals, obtain a considerable amount of the vitamin through bacterial synthesis in the intestinal tract.[35] It is therefore difficult to establish any exact specie requirements for this vitamin.

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Sydenstricker et al.[28] produced typical biotin deficiency symptoms in man in a few weeks by feeding large amounts of raw egg white (thirty per cent of daily caloric intake). The symptoms observed included; characteristic exfoliative dermatitis, pronounced pallor, mental depression, and general lassitude. All of these symptoms were rapidly cured (three to five days) by the adminis-

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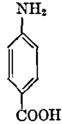
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Chapter 13

Para-Aminobenzoic Acid

(Achromotrichial Factor)

WHILE studying the toxicity of hydroquinone (in cats) Oettel[1] observed a distinct graying of the fur and thus was probably the first investigator to produce experimental achromotrichia. Nielsen et al.[2] reported the concentration and fractionation of a factor of the B complex which prevented achromotrichia in piebald rats. Since the most purified fraction contained only small amounts of pantothenic acid and since the crystalline material subsequently obtained was very active and distinct from the well recognized vitamins, the authors postulated the presence of a new factor of the B complex—a preventive of nutritional achromotrichia.



Para-Aminobenzoic Acid

Martin and Ansbacher[3] confirmed the work of Oettel and showed, by experiments with young mice, that the achromotrichia which developed in a period of four to twenty weeks was cured by feeding a rice polish concentrate or small amounts of para-aminobenzoic acid. Martin and Ansbacher suggested that the achromotrichia produced by hydroquinone was a vitamin deficiency and para-aminobenzoic acid was a factor of the B complex. Ansbacher[4] also demonstrated that para-aminobenzoic acid was an essential growth nutrient for chicks and required by rats for growth and maintenance of normal fur.

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used in the treatment of certain rickettsial infections and led Yoemans et al.[12] to use it in the treatment of 20 cases of louse borne typhus fever. The study showed that the early administration of PABA favorably modified the course of the disease. Smith[13] also reported favorable effects of PABA in the treatment of endemic (murine) typhus. The report of Rose et al.,[14] probably the first on the use of PABA in the treatment of Rocky Mountain Spotted Fever, was followed by the studies of Flinn et al.,[15] Best et al.,[16] Ravenel,[17] Tichenor et al.,[18] Muntz and Salkin[19] and others. Although important at the time (for the treatment of the above diseases) PABA has given way to the more effective, recently discovered antibiotics, aureomycin, terramycin and chloramphenicol.

Tauber and Laufer[20] have proposed a colorimetric method for the determination of para-aminobenzoic acid. Amino acids, urea, pantothenic acid, nicotinic acid or thiamine, do not interfere with the procedure.

Para-aminobenzoic acid appears to be widely distributed, occurring free and combined, in both plant and animal tissues.[21,22] No quantitative data are available. Little or nothing is known about the physiological action of PABA. Both man and animals excrete excesses of the substance, as the acetate, via the urine.[23] Very little is known concerning requirements of man and animals for this substance. In addition to the graying of the hair (fur) of animals produced by a deficiency of para-aminobenzoic acid, it has been pointed out in the preceding pages that deficiencies of biotin or pantothenic acid may also produce changes in the pigmentation of the hair (fur) of several species (mouse, rat, dog, fox). Although achromitrichia may be readily produced in animals and rapidly treated by the three vitamins just mentioned, they appear to be ineffective in man.

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Woods[5] found that certain yeast extracts contained a substance which reversed the inhibitory action of sulfanilamide on the growth of hemolytic streptococci. Since para-aminobenzoic acid showed similar effects, Woods felt the activity of the yeast fraction was due to this substance. Rubbo and Gillespie[6] found that para-aminobenzoic acid and certain yeast concentrates were required by *Cl. acetobutylicum* for growth and after removal of para-aminobenzoic acid the yeast concentrates were no longer active. Landy and Wyeno[7] found that para-aminobenzoic acid inhibited, *in vitro*, the bacteriostatic properties of sulfanilamide, sulfapyridine and sulfathiazole on streptococci, pneumococci and staphylococci.

Several investigators found that para-aminobenzoic acid (PABA) was one of the many substances which inhibited the production of the thyroid hormone and as a result produced hyperplasia of the thyroid gland and lowered the basal metabolic rate. Para-aminobenzoic acid was in the list of 106 compounds which Astwood[8] tested and found active (200 mg. per 100 Gm. of ration) in producing hyperplasia of the thyroid gland and a lowering of the basal metabolic rate. The action is primarily concerned with the inhibition of thyroid hormone production. The Mackenzies[9] observed that the hyperemia and enlargement of the thyroid gland, as well as the lowering of the basal metabolic rate produced by the incorporation of various sulfonamides in the diet of rats, were not prevented by several dietary factors including para-aminobenzoic acid.

Gordon et al.[10] fed rats a diet containing three per cent of para-aminobenzoic acid (PABA) to rats for 19–45 days and produced marked thyroid hyperplasia. Under the conditions of the experiments PABA had no effect on growth or on hematopoiesis. Sullivan and Archdeacon[11] administered intraperitoneally 7.5 mg. of PABA daily to male rats for 48 days. This amount of PABA produced inhibition of growth and definite toxic effects in all animals. There was marked distention (by gas) affecting the entire intestinal tract of all animals, and was so great as to interfere with the functioning of the abdominal organs (liver, spleen, etc.).

The several *in vitro* studies suggested that PABA might be

used in the treatment of certain rickettsial infec
Yoemans et al.[12] to use it in the treatment of 20
borne typhus fever. The study showed that the earl
tion of PABA favorably modified the course of
Smith[13] also reported favorable effects of PABA in th
of endemic (murine) typhus. The report of Rose et al.
the first on the use of PABA in the treatment of Rocky
Spotted Fever, was followed by the studies of Flin
Best et al.,[16] Ravenel,[17] Tichenor et al.,[18] Muntz and Sa
others. Although important at the time (for the treatm
above diseases) PABA has given way to the more
recently discovered antibiotics, aureomycin, terramy
chloramphenicol.

Tauber and Laufer[20] have proposed a colorimetric met
the determination of para-aminobenzoic acid. Amino
urea, pantothenic acid, nicotinic acid or thiamine, do not
fere with the procedure.

Para-aminobenzoic acid appears to be widely distrib
occurring free and combined, in both plant and animal tissue
No quantitative data are available. Little or nothing is kn
about the physiological action of PABA. Both man and ani
excrete excesses of the substance, as the acetate, via the urin
Very little is known concerning requirements of man and anim
or this substance. In addition to the graying of the hair (f
f animals produced by a deficiency of para-aminobenzoic ac
has been pointed out in the preceding pages that deficiencies
otin or pantothenic acid may also produce changes in the pig
entation of the hair (fur) of several species (mouse, rat, dog
). Although achromitrichia may be readily produced in
mals and rapidly treated by the three vitamins just mentioned,
appear to be ineffective in man.

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Chapter 14

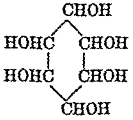
Inositol

(Mouse Anti-alopecia Factor)

INOSITOL, known to exist in many plant and animal tissues since discovered by Scherer in 1850, has only recently received attention from those engaged in the study of the nutrition of man, various animal species, and microorganisms.

Eastcott[1] prepared and studied a large variety of plant and animal extracts and showed that Bios I was identical with *i*-inositol and that it was required by yeast for maximal growth.

Inositol, chemically speaking is hexahydroxy cyclohexane. Theoretically there can be 8 stereoisomers—however only the inactive *meso*-form possesses biological activity.



Inositol

In 1940 Woolley[2] found that young mice, maintained on a purified diet supplemented with all of the known crystalline vitamins, required an unknown nutrient for growth and maintenance of normal hair. The hair changes were suggestive of a pantothenic acid deficiency, but since liver extract, a good source of pantothenic acid was not curative, the identity of the two factors seemed unlikely. Since curative tests with biotin concentrates and para-aminobenzoic acid were also negative, Woolley postulated the existence of a new vitamin and, for lack of a more descriptive name, suggested the term, "mouse anti-alopecia factor." Since the addition of phytin (from grain) or inositol (from liver)

resulted in gains in weight and complete restoration of the hair, Woolley[3] suggested that inositol, or its phosphoric acid ester, was the new factor.

Williams[4] produced nutritional achromotrichia in rats which was neither prevented nor cured by the administration of pantothenic acid concentrates or pure pantothenic acid. Dimick and Lepp[5] found that synthetic pantothenic acid cured and prevented an experimentally produced nutritional dermatitis in chicks but did not represent the full growth effects of "filtrate factor." The administration of pantothenic acid to rats promoted growth, cured the eye lesions ("spectacle eye condition"), improved the dermatitis and modified, but did not completely restore the color of the hair. Both groups expressed the opinion that rice bran concentrates contained a new factor required by the chick and rat for growth and also by the rat for the maintenance of normal color of the hair.

Norris and Hauschildt[6] found that mice on a certain purified diet failed to grow and developed skin lesions. The authors stated that the mouse required a new factor, present in yeast and liver, but not identical with thiamine, riboflavin, nicotinic acid, pyridoxine or "filtrate factor." In subsequent experiments Woolley[7] demonstrated that the growth of hair of mice was definitely influenced by inositol or its esters and also that inositol occurs in mammalian liver in combined form from which acid or alkaline hydrolysis yields inositol. In other experiments Woolley[8] showed that inositol is synthesized in the intestinal tract of mice and that the presence of pantothenic acid in the ration was important for such synthesis.

Pavcek and Baum[9] reported that 10 mg. of inositol per rat per day rapidly restored hair, cured the eye lesions ("spectacle eye condition") and augmented growth of rats. Cunha et al.[10] also observed a growth response in rats receiving inositol supplements and also found that it prevented the generalized, extensive loss of hair.

Several years ago Hogan and Ritchie[11] found that rabbits could be reared with normal growth rate on simplified rations and that, except for vitamin C, the same rations were adequate for growth and maintenance for the guinea pig. In later experiments,

Hogan and Hamilton[12] observed added growth response when guinea pigs were given a daily supplement of 10 mg. of inositol.

Cooperman et al.[13] reported that the hamster does not grow or survive long on synthetic diets which afford good growth and maintenance for the rat. Hamilton and Hogan[14] observed that 80 per cent of the hamster young were born undeveloped on an inositol-free diet. From the growth responses of eleven different groups of hamsters, maintained on the same basal diet and receiving various vitamin supplements, Granados and Dam[15] concluded that the hamster does not require dietary inositol, biotin, para-aminobenzoic acid, niacin, folic acid or vitamin K for growth. Choline appeared to produce an extra growth response. Hegsted et al.[16] *obtained good growth response in chicks* when 0.1 per cent of inositol was added to various rations, the response varying with the amount of inositol added to the ration. Jukes et al.[17] found that inositol deficiency resulted in slow growth of young turkeys and the development of a normocytic anemia. *The addition of one gram of inositol per 100 grams of ration pro*duced a good growth response.

Gavin and McHenry[18] found that inositol possessed lipotropic properties; in the rat it prevented the development of an acutely fatty liver and also the accumulation of cholesterol in the liver. *The lipotropic effects of inositol in rats has been confirmed* by Engel,[19] Forbes[20] and Handler.[21] Forbes also demonstrated that the lipotropic action of inositol was not dependent upon the presence of biotin as originally suggested by Gavin and McHenry[18] and that the lipotropic effects of choline plus inositol were *greater than either alone. MacFarland and McHenry*[22] subsequently used pure biotin, in place of the biotin concentrates and found the original concept concerning the role of biotin was erroneous. Handler[21] found that fatty livers developed in the rats receiving a basal B vitamin supplement, biotin and choline. *Good growth and an increase in food consumption* were noted. Increasing the folic acid content of the supplement accentuated the deposition of fat, growth rate and food consumption but only in the presence of biotin. Inositol was found to prevent the accumulation of fat in the liver and without any effect on growth or *appetite.* In *contrast* to this work, Eilert and Dragstedt[23]

found that 164 mg. of inositol per rat per day did not prevent development of fatty livers in rats receiving a high fat, low protein, yeast-supplemented diet.

Abels et al.[24] observed that the livers of patients with gastrointestinal carcinoma almost always contained excessive amounts of fat and that such fatty infiltration rapidly disappeared after the administration of "lipocaic." Further studies on patients indicated that the lipotropic properties of "lipocaic" were dependent upon the content of inositol. Herrmann[25] used either choline or inositol in the treatment of patients with atheromatous coronary disease and found that both of these lipotropic agents caused about the same lowering (17 to 20 per cent) of total blood cholesterol and cholesterol esters in these patients.

When rats were given a single dose of 200 mg. of inositol (in aqueous solution) it required 24 to 28 hours for complete absorption and urinary excretion did not account for one per cent of the amount ingested. Although the rat slowly utilizes inositol and no increase in liver glycogen could be demonstrated, Wiebelhaus et al.[26] believe the rat metabolizes inositol as carbohydrate. This view is supported by the fact that the ingestion of inositol alleviates ketosis and reduces total urinary nitrogen. Further proof of the conversion of inositol to glucose is presented by Stetten and Stetten[27] who administered *meso*-inositol containing an excess of deuterium to phlorhizinized rats and found significant amounts of deuterium in the glucose excreted in the urine. Many years prior to these observations, Greenwald and Weiss[28] administered inositol orally to phlorhizinized dogs and found a definite increase in the glucose:nitrogen ratio in each experiment and concluded that the dog slowly and incompletely converts inositol to glucose.

The requirements of man and various animal species for inositol remain to be established.

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Chapter 15

Choline, Betaine and Methionine

(The Essential Methyl Transfer Group)

ONE OF THE earliest references to methyl transfer is contained in the work of Thompson[1] who, from his studies, concluded that the *in vivo* synthesis of creatine was the result of methyl transfer. Allan and co-workers[2] observed that many of their diabetic animals (experimental), maintained in good condition for long periods by insulin, developed symptoms which terminated fatally. Autopsy showed extensive fatty infiltration of the livers of the animals. More than 30 years after Thompson[1] suggested the relationship between the *in vivo* synthesis of creatine and methyl transfer, Borsook and Dubnoff,[3] also du Vigneaud and co-workers,[4] proved that methyl transfer was involved in the *in vivo* synthesis of creatine and suggested that the transferable methyl group should be considered as an essential dietary component.[4] Best et al.[5a,5b,5c] showed that a deficiency of choline, betaine or methionine in the diet of young rats and dogs resulted in extensive fatty infiltration of the livers of the experimental animals. The livers of the deficient animals also showed an increase in the content of cholesterol esters,[6,7] and a diffuse nodular cirrhosis.[8a,8b,9,10] The kidneys, especially the cortex, are the site of hemorrhagic degenerative changes.[11a,11b] Best et al.[5a,5b,5c] demonstrated that the addition of choline or betaine to a diet rich in fat prevented the deposition of fat in the livers of the experimental animals and, about the same time, Tucker and Eckstein[12a,12b] showed that methionine also prevented the deposition of fat in choline-deficient animals. Previous studies had indicated that dogs did not survive on a purified diet supplemented with synthetic thiamine, riboflavin, nicotinic acid, pyridoxine and pantothenic acid and Schaefer et al.[13] showed that young puppies also require

choline for growth. Baxter[14] found that the severe illness and mortality of young rats on a choline deficient diet (the hemorrhagic kidney syndrome) were markedly reduced when corn starch was substituted for the sucrose in the diet. Although corn starch contains considerable choline (13 to 15 mg. choline chloride per 100 Gm.), extracted starch was equally effective as the untreated. Griffith[11c] had also observed that starch prevented the development of hemorrhagic kidneys in experimental animals. Baxter[14] found that the inclusion of 2 per cent of succinylsulfathiazole to the starch-containing diet did not alter the protective action of the starch and was unable to demonstrate that starch increased the synthesis of choline by the bacteria present in the intestinal tract.

Past work indicates that the nutritional functions of choline should be considered from two viewpoints. In the first category we have the function which depends upon the utilization of the intact molecule;[15] this includes the effects of choline in preventing fatty livers, hemorrhagic kidneys in mice and rats, and perosis in chicks. One should also include its rôle in lecithin (phospholipid) formation.[16] The second physiological action of choline depends upon the ability to provide a labile transferable methyl group.

Ever since the development of the general concept of "labile" methyl groups in mammalian metabolism it has been apparent that choline was one of the natural sources of such methyl groups. Two lines of evidence support such a view: (1) The work of Simmonds et al.[17] in which the feeding of choline labeled with deuterium in the methyl group led to the appearance of the labeled methyl groups in methionine and creatine isolated from the tissues of the experimental animals; and (2) the work on du Vigneaud et al.[18a,18b] which showed that young rats grow normally on a methionine-free diet containing adequate supplements of choline and homocystine. Borsook and Dubnoff[19] indicated that the formation of methionine from choline or betaine and homocysteine was one type of transmethylation. Although Borsook and Dubnoff thought their experimental evidence indicated that choline was a direct donor of methyl groups, they also noted that betaine was more effective than choline in the formation of methionine. More recent work[20] of these investigators places

considerable doubt upon the ability of choline to directly transfer its methyl group. Using the *in vitro* system of synthesis,[20] Muntz[21] prepared choline chloride containing an excess of N^{15} and allowed it to react with homocysteine in the presence of rat liver homogenate and found that the N^{15} appeared in a fraction possessing the characteristics of dimethylglycine. This clearly indicated that choline does not directly transfer the one labile methyl group but is first oxidized to betaine which becomes the direct donor. Welch and Sakami[22] have recently reported that repeated injection of C^{14} formate into rats resulted in the conversion of formic acid to labile methyl and isolated both methionine and choline after rat liver slices were incubated in the presence of C^{14} formate, homocysteine, dimethylaminoethanol, folic acid and vitamin B_{12}. After feeding methionine, with labeled methyl group to chicks, Burke et al.[23] isolated choline containing C^{14} from the carcasses. This work apparently brings the chick into conformity with man, the rat and the pig.

Betaine like choline supports growth of young on homocystine diets, it acts as a lipotropic factor and is able to prevent the development of hèmorrhagic kidneys.[11b] In growth experiments there is a lag of several days before betaine initiates growth in animals deficient in methyl groups but once started, the effects of choline and betaine are about equal. The work of Griffith and Mulford[11c] showed that on low concentrations in the diet, betaine was about one-third as effective as choline in preventing the hemorrhagic kidney syndrome. This corresponds to the presence of one labile methyl group in the betaine molecule. Jukes and Welch[24] showed that neither betaine nor methionine was effective in preventing perosis in choline-deficient chicks. Almquist and Grau[25] found that when small amounts of sulfur-bearing amino acids were included in the diet, betaine may function as a substitute for choline in chick nutrition.

Certain proteins may serve as a source of the transferable methyl group, the list includes the proteins containing methionine and those supplying the amino acids necessary for the *in vivo* synthesis of betaine.[26b] Not all methyl groups attached to quaternary nitrogen are transferable and the methyl group must be bound to a specific structure; for example, the methyl groups of

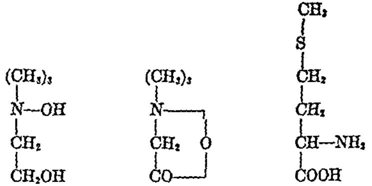
creatine,[4] S-methyl-cysteine[27a,27b] or of betaines from threonine and serine[28] are not transferable. Welch[27a] also showed that choline derivatives containing other alkyl groups, such as the methyl-diethyl homolog, prevent the deposition of fat in the liver[29] and the kidney hemorrhages,[27a] but do not support growth of rats maintained on a diet containing homocystine. Arsenocholine prevents the development of hemorrhagic kidneys in rats, perosis in turkeys, and the deposition of fat in the liver[30] but does not transfer the methyl group to homocysteine.[27a]

The favorable results (fall in blood sugar level, gain in weight) obtained from the use of choline in 26 cases of diabetes mellitus by Pelner et al.[31] prompted Gates[32] to repeat the work with a much larger number of patients. Gates found that the administration of 1 Gm. of choline chloride four times daily for an average of 42 days had no significant effect upon the severity of the disease, or the blood sugar levels, nor were there any observable general benefits from such therapy. Toxicity to choline was noted in nine out of the 100 patients (11 per cent); three cases developed severe diarrhea, and severe edema of lower extremities developed in five patients. All untoward symptoms rapidly cleared up when the drug was stopped.

The experimental production of cirrhosis in dogs, rats and rabbits by prolonged feeding of a diet low in choline, quite naturally led to the use of choline in the treatment of cirrhosis in man. The results of the clinical studies by Brown,[33] Russakoff and Blumberg,[34] Goldstein and Rosahn,[35] Beams,[36] Man et al.[37] were not consistent but when one views the complex nature of the disease it is not surprising that a single dietary component was not uniformly or completely effective. The clinical work suggested that choline may well be a valuable adjunct in the treatment of conditions causing the deposition of excess fat in the liver.

Both choline and methionine have been used, in varying doses, by a number of investigators[38,39,40] in the treatment of infectious hepatitis and again the evidence was quite contradictory. However a review of published clinical work indicates that neither choline nor methionine was of any appreciable value in the treatment of the deranged liver functions encountered in infectious hepatitis.

Choline is a colorless, viscous, strongly alkaline liquid. It rapidly absorbs moisture and carbon dioxide from the air. It is very soluble in water and alcohol but insoluble in ether. Choline readily forms salts (chloride, citrate, borate) which are usually white, hygroscopic crystals, and readily soluble in water. Betaine occurs as colorless deliquescent crystals which are readily soluble in water and alcohol but sparing soluble in ether. At 100°C. the compound loses its water, the anhydride melts at 293°C. with rearrangement. Methionine is optically active, $[\alpha]_D^{25} = 6.87°$ in water. The melting point, 283°C., is not sharp due to decomposition.



Choline Betaine Methionine

Neuman and Hodge[41] found the L.D.50 for rats was 3.4 to 6.1 Gm. of choline per kilo body weight. Melass et al.[42] found that the inclusion of 1 to 2 per cent of choline in the diet of chicks led to a lowered growth rate (about 13 per cent) and when 4 per cent of choline was added there was a more marked depression of growth (24 per cent). It has been reported[43] that the daily ingestion of 8 to 10 mg. of choline by dogs caused a fall in hemoglobin and in erythrocyte counts. McKibbin[44] determined the total lipid nitrogen and phosphorus content of different tissues obtained from choline-deficient puppies and littermate controls. Total liver lipid phosphorus and nitrogen remained the same in both groups. The livers of the deficient animals showed a significant decrease in lecithins and blood plasma of these same animals showed a marked drop, 33 per cent, in total lipid phosphorus and nitrogen. The lipid concentration of many tissues (lung, cerebrum, spleen, pancreas, kidney) remained constant

notwithstanding the severe deficiency. Greenberg and Rinehart[45] produced choline deficiency in monkeys by substituting alpha-protein of soy beans for dietary casein. The deficiency was characterized by diarrhea, loss of weight and by a significant decrease in the free and total choline of the blood plasma. Following the administration of large excess of choline, Luecke et al.[46] found that sheep and dogs excreted only a small amount (0.5 to 2.5 per cent) in the urine during 24 hours. The levels of free and total choline in the plasma, the concentration in the liver or in the kidney were not increased by feeding a large excess of choline to sheep. Johnson et al.[47] found that human subjects also excreted very small amounts of choline via the urine (0.7 to 1.5 per cent in 24 hours).

Methionine like choline has a dual role in the nutrition of the rat. First it is required for the building of tissue (*per se* or as a precursor of cystine) and secondly it serves as a source of labile methyl group for the formation of choline and possibly for the methylation of other compounds. Treadwell[48] attempted to determine the amount of methionine required by rats for: (1) growth; and (2) for the prevention of excessive deposition of fat in the liver. It was thought that optimal growth was afforded by 0.6 per cent of methionine but extra growth response was observed when the diet contained one per cent of methionine. The fat content of the liver steadily decreased as methionine was increased and the fat content of the liver reached normal values when the diet contained 1.2 per cent of methionine. Under the conditions of these experiments it was not possible to differentiate the lipotropic effects of methionine from its ability to promote growth.

Beveridge et al.[49] pointed out that the recorded observations showed the effects of diet upon liver fat and emphasized the nutritional importance of essential amino acids. In nutritional studies with rats, especially those involving low-protein diets, it is important to supply adequate amounts of choline (0.2 per cent of diet), otherwise an acute choline deficiency may be superimposed on a deficiency of essential amino acids.

Choline, or a compound furnishing transferable methyl groups, is required by dogs, rats, rabbits, guinea pigs, chicks and turkeys.

It seems to be concerned with growth, so the requirements a greater for the young than for the adult. Choline has been four essential for the growth of the *Pneumococcus bacillus*[50] and it ma be necessary for other microorganisms. Very little can be state regarding different specie requirements: dogs need about 35 m per kilo body weight;[51] rats require 10 to 20 mg. per day to en sure normal fat deposition;[52] and chicks require about 75 m per day.[53]

Glick[54] and Entenman et al.,[55] Winzler and Meserve[56] have applie the reineckate method and subsequent ultra-violet absorption a 327 mμ. to the estimation of choline in natural materials. Appl ton et al.[57] recently proposed a method whereby choline is pr cipitated as an iodine complex and subsequently estimated b absorption at 365 mμ. The method may be used to determin choline in urine, plasma phospholipids and general biologic material. Horowitz and Beadle[58] suggested a microbiologic method using neurospora as the assay organism.

Pork liver, beef liver, egg yolk, defatted wheat germ, live sausage, peanuts and peanut butter are good food sources c choline.[59]

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Chapter 16

Laevo-Ascorbic Acid, Dehydro-Ascorbic Acid

(Vitamin C)

It is doubtful whether most readers realize that for centuries scurvy occurred frequently in all classes of people: sailors, explorers, armies in the field and "stay-at-homes." More than 200 years ago it was found that scurvy, the disease of sore gums, painful joints and hemorrhages, could be cured by the addition of fresh vegetables to the diet or by the administration of small quantities of fruit juices. The early accounts, not available to the writer, are referred to frequently by Harris.[1] In the chapter on scurvy it is stated that the earliest account of scurvy dates back to the time of the Crusades. The havoc caused by scurvy amongst sailors can be inferred from the following excerpt from the book by Harris[1]: "Admiral Sir Richard Hawkins mentioned, in 1593, that 10,000 men had died from scurvy within his own personal experience" and "when Vasco da Gama sailed around the Cape of Good Hope in 1498, 100 of his men out of a crew of 160 perished from scurvy. During Cartier's second voyage to Newfoundland in 1535, scurvy broke out and soon 100 of his 103 men were very sick and 25 died." One of the earliest accounts of scurvy was Lind's famous book, published in 1757.. For his experiment he selected 12 sailors: two were completely cured by "two oranges and one lemon given everyday"; two others improved on a quart of cider per day; and the others, receiving various treatment became worse. In 1804, British Navy regulations required a daily ration of lemon (lime) juice. (Subsequently all of the slow sailing vessels were dubbed "lime juicers.") So many artifically fed infants developed scurvy that the "sailors' calamity" soon became a "babies' calamity." Infantile scurvy

was first delineated by Sir Thomas Barlow in 1883 and thus the condition was known for many years as "Barlow's Disease." Our modern approach to scurvy and its specific prevention and treatment is based on the discovery, by Holst and Fröhlich[2] in 1907, that scurvy could be produced experimentally in guinea pigs.

Vitamin C is a growth factor for many plants, hence is distributed very widely in the growing tissues. It is formed rapidly in sprouting seeds and reaches high concentrations in actively growing parts of the plant. The mechanism whereby C is formed in plants is unknown. The concentration of C in the growing leaf is a function of the light received and, therefore, fluctuates considerably. Parts of plants, which have become "woody," do not contain C. Dry seeds do not contain C *per se* but do contain a precursor, which is rapidly converted during the early stages of germination. According to King[3] there is no clear evidence to indicate the actual precursor of vitamin C in either plants or animal tissues. The common nutrients for plant and animals, that might possibly serve as precursors, are glucuronic and galacturonic acids. In favor of this assumption, it has been observed that dextrose increases the ascorbic acid content of slices of intestinal tissue.[4] Of all the sugars studied, both *in vitro* and *in vivo* and in experiments with plant[5] and animal tissues,[6a,6b] mannose produced the greatest increase in ascorbic acid. It has also been suggested that *l*-sorbose may serve as a precursor of vitamin C.[7] Rudra[8a,8b,8c] considers that traces of manganese are necessary for the synthesis, by plant and animal tissue slices, of ascorbic acid from mannose, galactose or dextrose. Rudra also suggested that the very low manganese content in human and guinea pig tissues might be associated with the inability of these two species to synthesize the vitamin. From the preparation and testing of the possible steroisomers it is evident[9] that full antiscorbutic activity requires the *d*-configuration at carbon 4 and a hydroxyl group at carbon 5 (see chemistry of vitamin C p. 221). A limited number of compounds[10,11] possess some vitamin C action, varying from 2 to 30 per cent of that possessed by ascorbic acid. Reedman and McHenry[12] demonstrated by chemical and bi

etry, the existence of an ascorbic acid-protein complex in certain plant tissues. The combined form was as effective an antiscorbutic agent as reduced *l*-ascorbic acid.

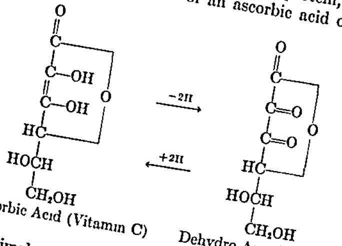
Three widely separated laboratories (Zilva, Bezssonoff and King) were actively engaged for a number of years in the isolation and identification of this vitamin. The earliest definite identification, made by King and Waugh,[13,14] was soon confirmed by Svirbely and Szent-Györgyi,[15a,15b] Tillmans and Hirsch.[16] Within a short time it was shown by these groups that the crystalline material isolated from lemons, oranges, cabbages and adrenal glands, and effective against scurvy, was a single substance and, as the term "hexuronic acid" implied, it was a derivative of a six-carbon-sugar.[17,18] Elaboration of the structure and synthesis of vitamin C were carried out in the laboratories of Haworth,[19] Karrer,[20] Von Euler,[21] Kraft,[22] and Reichstein.[23]

The chemical structure of ascorbic acid,

$$\text{(R)}-\underset{\text{OH}}{\underset{|}{\text{C}}}=\underset{\text{OH}}{\underset{|}{\text{C}}}-\underset{\text{O}}{\underset{\|}{\text{C}}}-\text{(R)},$$

places it in a class of substances generally known as "reductones," and all compounds in this general group form reversible oxidation-reduction systems. Pure *l*-ascorbic acid crystallizes in white, odorless plates melting at 190 to 192°C. The vitamin is readily soluble in water (1 Gm. in 3 cc.), moderately soluble in absolute alcohol or glycerine (1 Gm. in about 50 cc.). Although the crystalline vitamin is relatively stable, all solutions tend to oxidize, especially in the presence of air (oxygen), traces of iron or copper, and exposure to light.[24,25] Ascorbic acid is optically active, in water $[\alpha]_D^{20} = +23°$. The compound exhibits a typical absorption spectrum at 265 mμ.[26] It is a monocarboxylic acid and readily forms typical salts. Oxidation of ascorbic acid (with oxygen,[27] hydrogen peroxide, ferric chloride, copper salts,[28] iodine, etc.) forms dehydro-ascorbic acid, which, in turn, may be reduced (with HI, H_2S, etc.) to ascorbic acid. Hirst[29] has shown that at the moment of formation, the dehydro-ascorbic acid is actually a neutral compound, the lactone. This, however, hydrolyzes in water with the formation of a free carboxyl (COOH) group. Thus, the acidity of ascorbic acid is actually due to the enolic hydroxyl group.[29]

It seems plausible to assume the existence of various enzyme systems in the body tissues which provide for the reduction and oxidation of ascorbic acid. It has been shown that blood[30] and certain plant extracts[31] (juices) contain an enzyme or enzymes which bring about the reduction of dehydro-ascorbic acid. Glutathione and other compounds with sulfhydryl-groups (all effective reducing agents) serve as *in vitro* protective agents for vitamin C.[32] It has been easy to demonstrate the presence of a very active enzyme, "ascorbic acid oxidase," in plant tissues which readily oxidizes vitamin C to the dehydro-form.[33] Ascorbic acid oxidase is thought to be a copper protein,[33] specific for *l*-ascorbic acid. The presence of an ascorbic acid oxidizing en-



Ascorbic Acid (Vitamin C)   Dehydro-Ascorbic Acid

zyme in animal tissues is still questionable, however there is definite evidence for the oxidation of ascorbic acid by cytochrome oxidase plus cytochrome-C.[34] In addition to the function of transporting hydrogen in cellular respiration, vitamin C is intimately concerned, in some unknown way, with the formation of the intercellular colloidal substances; cartilage, dentine, bone matrix, the collagen of fibrous tissues and non-epithelial cement substances.[35,36] Thus abnormal calcium metabolism, the result of a deficiency of vitamin C, affects the growth and maintenance of teeth and bones and in many details the effects are similar to those produced by a deficiency of vitamin D. A mild, chronic form of C-deficiency frequently produces oral lesions which may be diagnosed as "pyorrhea."[37]

The *clinical* symptoms of C-deficiency (scurvy) are characterized by a hemorrhagic syndrome. Commonly, these hemorrhages occur at sites of greatest stress and where growth processes are most active.[38] During C-deficiency, the bones cease growing, normal junctions are replaced by connective tissue containing calcified cartilage rather than normal osteoid tissue. In the mouth, the gingiva become swollen and spongy and bleed easily. There are extensive changes in tooth structure, involving enamel, cementum and, most of all, the dentine. The newly formed structures are porotic and are lacking in strength. Boyle[39] found dentine was not formed during the late stages of acute scurvy (animals). From a study of teeth of children with hospital records of acute scurvy and from a study of tooth germs removed at autopsy, Boyle believes that although there is some arrest in dentine formation, human teeth are much less affected by vitamin C deficiency than are the teeth of experimental animals. Ample amounts of vitamin C should be provided to all cases of bone fractures and those recovering from major surgical operations.[40,41] Anemia, of varying severity, is almost a universal finding. In acute scurvy the vitamin should be administered parenterally in large amounts. Vitamin C deficiencies may occur as the result of intestinal tract disturbances[42] (ulcers) which interfere with absorption or conditions which accelerate destruction.[43]

To determine the status of vitamin C metabolism or to detect clinical vitamin C deficiency, one may make use of several procedures:

(1) determine the plasma content;[44]

(2) establish the daily urinary excretion before and after the administration of an established test dose;[45]

(3) x-ray examination of the bones, especially the end of the shaft of the long bones[46] (useful only in severe scurvy);

(4) the intradermal test;[47] and

(5) the capillary fragility test of Dalldorf.[48]

Following the ingestion of a test dose of vitamin C, the blood level reaches a maximum value about 1.5 hours after ingestion of a test dose of vitamin C and the excess is excreted in the urine within four to six hours.

Chemical methods depending principally on the reducing ac-

tion of ascorbic acid have replaced the old bioassay procedure. Since dehydro-ascorbic also acts as a vitamin and has no reducing capacity, the assay of natural products requires special care. Titration with 0.01 N iodine solution may be used to determine vitamin C in pure solutions but this method does not measure the naturally occurring dehydro-ascorbic acid, and other reducing substances frequently interfere with the endpoint. Titration with 2,6-dichloro-phenol-indophenol, originally proposed by Tillmans,[49] or some modification, is most widely used for determining the vitamin C present in foods, animal tissues, milk, etc.[50,51,52] In studies dealing with human nutrition one should keep in mind the procedure of Melnick et al. which determines physiological availability (see reference 75 under *Thiamine*).

Hamil et al.[53] studied the vitamin C requirement of 247 infants and came to the conclusion that the minimal protective dose for the average infant was 10 mg. of pure vitamin. From these studies the authors also concluded that the condition usually classified as latent scurvy was in reality mild scurvy, a condition which might exist for several months with alternate periods of clearing and reoccurrence, resulting in slower growth and general retardation of physiological processes.

From clinical experience and by inference from the ascorbic acid content of breast milk, Ingalls[54] suggested that small infants need 20 to 30 mg. per day (45 to 60 cc. of orange juice). Heinemann[55] regarded 0.8 mg. per kilo per day as the necessary intake for a health adult and 0.4 mg. or less per kilo to protect against scurvy. Ralli and co-workers[56a,56b] studied the vitamin C requirements of three adult males over a period of 110 to 240 days. At first each subject received 50 mg. daily, then 75 and on to 350 mg. On a daily intake of 100 mg. all three subjects attained plasma levels of 1.0 mg. or more per 100 cc. A daily intake of 50 mg. per day was not sufficient to maintain this level, plasma values dropped to 0.4 mg. per 100 cc. which the authors regard as the lower normal value. Minot et al.[57] made an extensive study of vitamin C serum values in a large number of children. Serum levels below 0.3 mg. per 100 cc. were indicative of frank deficiency. These investigators reported that approximately 50 per cent of the children studied, without indications of other diseases, were in an unsatis-

The clinical symptoms of C-deficiency (scurvy) are characterized by a hemorrhagic syndrome. Commonly, these hemorrhages occur at sites of greatest stress and where growth processes are most active.[38] During C-deficiency, the bones cease growing, normal junctions are replaced by connective tissue containing calcified cartilage rather than normal osteoid tissue. In the mouth, the gingiva become swollen and spongy and bleed easily. There are extensive changes in tooth structure, involving enamel, cementum and, most of all, the dentine. The newly formed structures are porotic and are lacking in strength. Boyle[39] found dentine was not formed during the late stages of acute scurvy (animals). From a study of teeth of children with hospital records of acute scurvy and from a study of tooth germs removed at autopsy, Boyle believes that although there is some arrest in dentine formation, human teeth are much less affected by vitamin C deficiency than are the teeth of experimental animals. Ample amounts of vitamin C should be provided to all cases of bone fractures and those recovering from major surgical operations.[40,41] Anemia, of varying severity, is almost a universal finding. In acute scurvy the vitamin should be administered parenterally in large amounts. Vitamin C deficiencies may occur as the result of intestinal tract disturbances[42] (ulcers) which interfere with absorption or conditions which accelerate destruction.[43]

To determine the status of vitamin C metabolism or to detect clinical vitamin C deficiency, one may make use of several procedures:

(1) determine the plasma content;[44]

(2) establish the daily urinary excretion before and after the administration of an established test dose;[45]

(3) x-ray examination of the bones, especially the end of the shaft of the long bones[46] (useful only in severe scurvy);

(4) the intradermal test;[47] and

(5) the capillary fragility test of Dalldorf.[48]

Following the ingestion of a test dose of vitamin C, the blood level reaches a maximum value about 1.5 hours after ingestion of a test dose of vitamin C and the excess is excreted in the urine within four to six hours.

Chemical methods depending principally on the reducing ac-

tion of ascorbic acid have replaced the old bioassay procedure. Since dehydro-ascorbic also acts as a vitamin and has no reducing capacity, the assay of natural products requires special care. Titration with 0.01 N iodine solution may be used to determine vitamin C in pure solutions but this method does not measure the naturally occurring dehydro-ascorbic acid, and other reducing substances frequently interfere with the endpoint. Titration with 2,6-dichloro-phenol-indophenol, originally proposed by Tillmans,[49] or some modification, is most widely used for determining the vitamin C present in foods, animal tissues, milk, etc.[50,51,52] In studies dealing with human nutrition one should keep in mind the procedure of Melnick et al. which determines physiological availability (see reference 75 under *Thiamine*).

Hamil et al.[53] studied the vitamin C requirement of 247 infants and came to the conclusion that the minimal protective dose for the average infant was 10 mg. of pure vitamin. From these studies the authors also concluded that the condition usually classified as latent scurvy was in reality mild scurvy, a condition which might exist for several months with alternate periods of clearing and reoccurrence, resulting in slower growth and general retardation of physiological processes.

From clinical experience and by inference from the ascorbic acid content of breast milk, Ingalls[54] suggested that small infants need 20 to 30 mg. per day (45 to 60 cc. of orange juice). Heinemann[55] regarded 0.8 mg. per kilo per day as the necessary intake for a health adult and 0.4 mg. or less per kilo to protect against scurvy. Ralli and co-workers[56a 56b] studied the vitamin C requirements of three adult males over a period of 110 to 240 days. At first each subject received 50 mg. daily, then 75 and on to 350 mg. On a daily intake of 100 mg. all three subjects attained plasma levels of 1.0 mg. or more per 100 cc. A daily intake of 50 mg. per day was not sufficient to maintain this level, plasma values dropped to 0.4 mg. per 100 cc. which the authors regard as the lower normal value. Minot et al.[57] made an extensive study of vitamin C serum values in a large number of children. Serum levels below 0.3 mg. per 100 cc. were indicative of frank deficiency. These investigators reported that approximately 50 per cent of the children studied, without indications of other diseases, were in an unsatis-

factory state with respect to vitamin C. Fincke and Landquist[58] maintained four college students (two men and two women) on a constant diet which provided 8 to 11 mg. of ascorbic acid per day. For seven successive days each of the two women received 50 mg. supplement and each of the two men received 60 mg. The study showed that it required an average of 1.0 mg. per kilo, of ascorbic acid per day to maintain a plasma concentration of 0.8 mg. per 100 cc. These investigators regard plasma value a better criterion of tissue saturation than the estimation of excretion after a fixed test dose.

Bessey and White [59] studied the vitamin C requirement of 93 children (ages 5 to 13), with accurate dietary records and came to the conclusion that the 40 to 50 mg. of ascorbic acid was an adequate daily intake for children in the indicated group. Roberts et al.[60] studied blood levels and urinary excretion of a test dose as means of establishing nutritional levels in 30 girls (age 6 to 12). In no instance was it possible to maintain a plasma level of 0.7 mg. per 100 cc. on an intake of 32 or 42 mg. per day. This study indicated that the above age group requires 62 to 72 mg. of ascorbic acid per day. Storvick et al.[61] studied the ascorbic acid requirements of 81 adolescent children (age 11 to 18) and came to the conclusion that this particular age group required more ascorbic acid per kilo body weight than either younger children or adults. Haines et al.[62] studied the effect of three levels of ascorbic acid intake in 12 adults (six men, six women, age 20 to 46) and concluded that a daily intake of 70 mg. per day was barely sufficient to maintain tissue saturation in the persons studied. These investigators agree with Storvick et al.[61] that although the allowances proposed by the National Research Council[63] provided a wide margin of safety, in terms of the amount required to prevent scurvy, the amount is not sufficient to maintain tissue saturation.

Roberts and Roberts[64] studied five children between the ages of 7 and 12, on various levels of intake, from 55 to 145 mg. per day and found, on the basis of a 50 per cent excretion of the 300 mg. test dose in 24 hours, that the two youngest required 65 mg. per day, the three oldest required 0.75 mg. per day. These levels of intake resulted in serum levels of more than 0.7 mg. per 100 cc.

Purinton and Schuck[65] found that more than half of their group of 63 adults had fasting plasma levels below 0.8 mg. per 100 cc. and recommended at least 100 mg. of ascorbic acid daily for young adults. Brown and co-workers[66] studied ascorbic acid metabolism in over 800 college students and found (on two non-consecutive mornings) that 23.7 per cent of the men (total 342) and 50.1 per cent (total 471) of the women had plasma values of 0.8 mg. or more per 100 cc. Students eating at home showed the highest C values.

From 1935 to 1945 Vilter et al.[67] studied 19 cases (18 men, 1 woman; age ranged from 16 to 84 years) of severe scurvy admitted to the Cincinnati General Hospital. Eleven of these patients were studied in detail. All but two of the 19 had anemia of normochromic, normocytic type. Persistent reticulocytosis, leucopenia and thrombopenia were commonly observed. Perifollicular hemorrhages, edematous blue-red tender gums and painful swollen joints were frequently seen. In each case there was prompt clinical and hematological improvement following the administration of vitamin C.

Wright[68] pointed out the large variations reported for human requirements of vitamin C and stated that as much as 100 mg. daily by mouth failed to cure scurvy. This failure was no doubt due to an inability to absorb from the intestinal tract, as parenterally administered vitamin C cured the scorbutic condition. Wright also reported that he had given as much as ten grams of the vitamin in a single intravenous dose without any untoward effects. Also gave one gram daily, intravenously, for many months without evidence of toxic side-actions.

Loeffler[69] studied the retention of ascorbic acid in large scale commercial freezing of red raspberries (15 to 20 million pounds per year). Fresh mature berries contain 20 to 27 mg. of vitamin C per 100 gms. and essentially all of the vitamin was retained during commercial freezing and during a storage period of 120 days at 23°C. Storage for 28 months caused about 25 per cent loss of the vitamin C content. The incorporation of sugar, as 40 to 50 per cent syrup, protects vitamin C. Lee et al.[70] found no loss of ascorbic acid during the slow or quick freezing of peas and snap beans.

Holmes et al.[71] found that raw milk, average of 32 samples, contained 19.7 mg. of ascorbic acid per liter. After controlled pasteurization the milk retained 15.9 mg. (80 per cent) of the vitamin. Stewart and Sharp[72] found very little data on the amount of vitamin C present in milk and commercial milk products sold to consumers and pointed out that many writers have *failed to take into consideration* the dehydro-ascorbic acid.

Stewart and Sharp[72] determined the total vitamin C content of 364 samples of pasteurized milk in the San Francisco, Oakland, and Berkeley region during the summer months of 1941 and 1942. Ten milk plants supplying 80 per cent of market milk were included in the study; the samples included 237 quart cartons sold in stores and 127 glass bottles delivered to the home, the study therefore represents home consumption conditions. The average of the 364 samples were: reduced ascorbic 3.4 mg. per liter, dehydro-ascorbic acid 2.4 mg. per liter. There were marked variations in both reduced ascorbic and dehydro-ascorbic acid content *but the sum of the two was quite constant. To further simulate home conditions*, 110 samples (44 bottles and 66 cartons) were held an additional 24 hours in an electric refrigerator at approximately 10°C. *Analysis showed that such holding caused a* 30 per cent loss in total vitamin C content. Twenty five samples of 12 major brands of evaporated milk were tested and found low in both forms, average total of 4.0 mg. per liter. 2890 samples of spray process powdered whole milk were examined; the average total C content (most in reduced form) was 12.3 mg. per 125 gms. of powdered milk (the amount used in making 1 liter of reconstituted milk). One pound tins of powdered milk, stored at room temperature (22°C.) for 3.6 and 12 months, lost 20 per cent in 12 months while 1050 samples of raw milk, analyzed at 15 different periods, representing various producers, had an average of 17.1 mg. of reduced ascorbic acid per liter.

Ingalls et al.[73] found that fresh breast milk contained an average of 4.5 mg. per 100 cc. and estimated that a normal, nursing infant ingested an average of 28 mg. of C per day. The pasteurization of fresh breast milk results in the loss of 93 per cent of the ascorbic acid (only 0.3 mg. per 100 cc.). There is apparently a mechanism which furnishes the foetus with an adequate amount

of vitamin C during the gestational period, even though the diet of the mother is relatively deficient in the vitamin. This same mechanism, or possibly a counterpart, also provides the nursing infant with an adequate supply of ascorbic acid. Pasteurized and evaporated milk are poor sources of vitamin C and infants and growing children require supplements, either in the form of citrus fruits and juices or pure vitamin C.

Guerrant et al.[74] made extensive studies of canning operations in New York, Delaware and Maryland during the 1943 and 1944 seasons. Except for tomatoes and tomato juice it was found that the various processes (blanching, sterilizing) caused 40 to 60 per cent loss of ascorbic acid. In a previous study Guerrant et al.[75] reported that tomato juice, stored for one year at 30°F., retained 92 per cent of the original ascorbic acid content. Under the same conditions, green lima beans lost 17 per cent. Holmes et al.[76] made a survey of the vitamin C content of fresh and canned fruit juices. To supply 50 mg. of ascorbic acid it requires the following amounts of fresh fruit juice: grapefruit 106 cc.; lemon, 107 cc.; orange 167 cc.; tomato, 368 cc.; pineapple, 568 cc. The canning industry now recognizes the large losses of vitamin C caused by oxidation and boil all cooking water to exclude oxygen.

Sutherland et al.[77] studied vitamin retention in foods cooked by household ways (open pan) and by the usual institutional procedure (open, steam jacketed kettles). When cooked in an open sauce pan, cabbage and spinach retained 45 per cent; when cooked in a steam jacketed kettle 51 per cent was retained. Open pan cooking of spinach resulted in 75 per cent loss of C and open kettle cooking caused a loss of 67 per cent of the C originally present. Alexander and Feaster[78] studied the effects of canning on the vitamin C retention of peas. In 1941, 24 samples of raw peas contained an average of 25 mg. per 100 G. In 1942, 22 samples of raw peas averaged 35 mg. of C per 100 Gm. Forty-five samples of peas canned in 1940–41 showed an average C content of 9.6 mg. per 100 Gm. (entire contents), approximately one-third of the C was present in the brine usually discarded! Noble and Hanig[79] determined the ascorbic acid and dehydro-ascorbic acid present in four fresh market lots of spinach, cauliflower, cabbage, asparagus and peas and determined losses in-

curred by different cooking procedures (pressure pan, tightly covered kettle and in a steamer). In general, and with a minimum amount of water, the cooked vegetables retained about 67 per cent of the C originally present. Van Duyne et al.[80] found that home cooking practices resulted in a loss of about 40 per cent of the vitamin C. Holding (in a refrigerator overnight) and reheating results in marked loss of C.

Heller et al.[81] studied vitamin losses during large scale handling and cooking of foods (frozen and raw vegetables) and found that careless handling during preparation, or excessive holding on steam tables, caused excessive losses of vitamin C, ranging from 27 to 90 per cent. Nagel and Harris[82] made an extensive survey of a college dining room which served about 600 persons per meal. The study was confined to an examination of nine vegetables; samples were taken of raw vegetable, after cooking (open kettle or pressure cooker) and after holding on a steam table for three hours. After cooking there was an average loss of 45 per cent of the ascorbic acid. Much of this loss was due to leaching by the cooking water. The authors suggest that those dependent, upon hotel and restaurant cooking should *eat early* and include liberal amounts of fresh fruits and vegetables (as in salads).

In a study dealing with stabilizers of ascorbic acid in fruits and vegetables, Hochberg et al.[83] showed that ascorbic acid oxidase which rapidly destroys ascorbic acid *in vitro*, is inactive in the digestive tract, of an adult man. Also the destructive action of copper is not observed in the gastrointestinal tract of man. From well controlled studies on man the authors show that ascorbic acid is more stable in natural fruit juices than in aqueous solution of the pure vitamin. The stabilizing factors in the natural juices are not effective in the gut, and do not increase the availability of ascorbic acid. It was found that apple juice is an excellent vehicle for ascorbic acid and that fortified apple juice retains potency for long periods.

The various studies on the retention of vitamin C (total) in processing and storage of foods, and the disastrous effects of almost every type of cooking indicate that man must rely upon fresh raw fruits and vegetables for an adequate supply of this important vitamin.

Some of the generally available foodstuffs rich in v
citrus fruits (orange, grapefruit, pineapple) and
berries, raspberries, tomatoes, cantaloupe, banana
green leafy vegetables, snap beans and peas.

The more liberal and optimal amount of B_1 (Nation
Council) needed to meet the demands of various
and different physiological states are shown in detai
II, page 242.

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Chapter 17

Flavones

(Vitamin P (Citrin) Rutin)

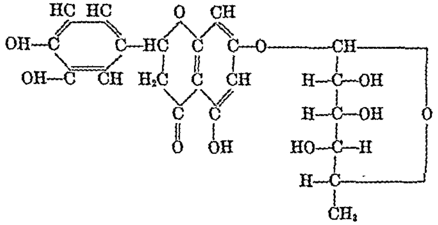
In 1936 Bentsáth, Rusznyák and Szent-Györgyi[1a,1b] reported that certain pathological lesions characterized by an increased permeability of the capillary wall, did not respond to treatment with vitamin C but were readily and completely cured by the administration of an extract of red peppers or lemon juice. The authors postulated that cellular vitamin C was accompanied by a substance of similar importance and related activity. In the absence of both vitamin C and the second substance the symptoms of scurvy predominated and obscured the symptoms caused by a deficiency of the new factor. Since the new material was closely associated with capillary permeability, the term "P" was suggested. Fractionation of red pepper or lemon juice concentrates provided very active material which was identified as a flavanone. In a subsequent report Bruckner and Szent-Györgyi[2] indicated that the active material was a mixture of hesperidin and eriodictyol glucosides. Zilva's experiments[3] with guinea pigs led him to disagree with the idea that the complete treatment of scurvy required both vitamin C and the new factor vitamin P. McHenry and Perry[4] were also unable to demonstrate that purified vitamin P had any prophylactic or curative effects on capillary fragility associated with experimental scurvy (guinea pigs). The "mixtures" originally mentioned by Szent-Györgyi have been the cause of considerable confusion in the chemical identification and also with respect to biological activity. Several investigators have endeavored to work out and clear up the chemistry of vitamin P and although crystalline material has been obtained, no one has been able to prepare pure eriodictin or hesperidin.

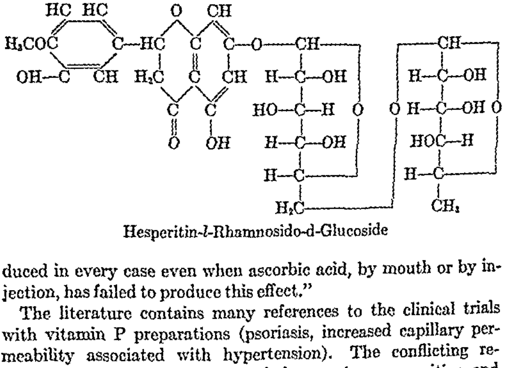
Thus Mager[5] isolated a light yellow, crystalline product which was identified as eriodictyol-*l*-rhamnoside, a glucoside which on

hydrolysis yielded crystalline eriodictyol, M.P.
Higby,[6] working with lemon peel, obtained: hespe
side-d-glucose (hesperidin), m.p. 261 to 262°C.
M.P. 291 to 292°C.; and eriodictin. By hydrolys
dictin Higby obtained crystalline eriodictyol, M.P.
the melting point, 250 to 260°C. found by Mager).
Webb[7] isolated microcrystalline material, M.P. 261
which, although insoluble in water, was biologically
it increased capillary resistance. The material was c
"hesperidin chalcone." When suspended in absolu
alcohol, with traces of hydrochloric acid, the compoun
reverted to hesperidin, M.P. 261 to 262°C. The cha
pared by Wilson[8] and that by Hibgy[6] was water soluble
very low melting point, 110 to 112°C. (with decomp
It seems quite evident that there are two active flavone
mins P"); the two glucosides, eriodictin (eriodictyol-*l*-r
side) and hesperidin (hesperitin-*l*-rhamnosido-d-glucose).
also apparent that biological activity is dependent upon th
ing of the flavanone rings to form the corresponding cha

Notwithstanding the contradictory literature as to the
ence of a factor (or factors) other than vitamin C required f
prevention and cure of certain hemorrhagic conditions (caus
nutritional deficiency), it would seem that the rat experimen
Rusznyák and Benkó[9] indicate the presence and need for
substance(s) referred to as "vitamin P." Scarborough[10]
lieves that it has been demonstrated beyond doubt that the m
terial referred to as "vitamin P" is effective in increasing capilla
resistance in experimental animals (rat, mouse, guinea pig) a
man. The term "vitamin P" or "citrin" refers to certain cry
talline flavone complexes, which through their sharp meltin
points and general similarity with pure substances have been th
cause of much confusion. In conclusion Scarborough states
"In human subjects suffering from multiple vitamin deficiency of
varying degrees of severity the capillary fragility was found to be
increased. This increase has been determined in spontaneous,
conditioned and induced deficiencies. There is present in orang
and lemon juices, and in certain extracts ma
substance (or substances) fla

crease the resistance of capillary walls to the application of pressure. The precise nature of the material producing this effect has not been determined. It is active when given by mouth, by intramuscular injection or through the rectum. The decrease in capillary fragility (or increased capillary resistance) has been pro-


Eriodictyol-*l*-Rhamnoside


Hesperitin-*l*-Rhamnosido-d-Glucoside

duced in every case even when ascorbic acid, by mouth or by injection, has failed to produce this effect."

The literature contains many references to the clinical trials with vitamin P preparations (psoriasis, increased capillary permeability associated with hypertension). The conflicting reports may well be a reflection of the varying composition and biological activity of the preparations used.

Results of experiments with 107 guinea pigs showed that a

combination of rutin and ascorbic acid, given simultaneously prolonged the life of scorbutic animals beyond that of guinea pigs receiving vitamin C, rutin or quercetin separately.[11] The work confirms the experiments reported by Rusznyák and Benkó,[9] and Scarborough[12a,12b] and shows that the syndrome of experimental scurvy is due to a deficiency of vitamin C and certain flavones (P, citrin, Hesperidin, eriodicyol).

Assuming a relationship between psoriasis, and injury, also between injury and increased fragility, Niedelman and Horoschak[14] treated 20 cases of established psoriasis (of eight years' duration) with hesperidin, 100 mg. four times daily. After four days the dose was increased to 600 mg. daily and continued for four months. In the group of twenty, one patient showed definite improvement, 12 showed no change and seven became worse. Each person in a second group of 20 cases of psoriasis received 50 mg. of hesperidin and 50 mg. of ascorbic acid four times daily. Twelve patients showed no change and five became worse. In a third group of 10 patients treated with ascorbic acid only (400 to 600 mg. daily) four showed no change and five became worse. Ten patients received placebos: two improved, and five became worse. These results were at variance with the observations of Goldfarb[13] who used a "citrin lemonade" and obtained marked improvement in 65 per cent of his cases. Griffith and Lindauer[15] found that about 18 per cent (of 265) of patients with hypertension exhibited increased capillary fragility and about 85 per cent of such cases were benefited by the administration of hesperidin or hesperidin chalcone.

Recently another flavanone, rutin, is replacing "vitamin P" in studies with increased capillary fragility.[16] Rutin, a crystalline glucoside of quercetin, may be obtained from many plant flowers and leaves (buckwheat leaves and blossoms, tomato stems, elderberry leaves, etc). Rutin, the rhamnoglucoside of quercetin, is a flavone derivative and in structure closely resembles hesperidin. Pure rutin (3,5,7,3′,4′-pentahydroxyflavone-3-rutinoside) is obtained in the form of bright yellow, needle-like crystals which melt at 192 to 196°C. (decomposition at 214°C.). It is soluble in methyl or ethyl alcohol, in acetone, ethyl acetate and alkali. It is insoluble in chloroform, ether and petroleum ether. Hydrolysis with dilute acids yields quercetin, glucose and rhamnose.

Griffith et al.[17] were probably the first to use rutin in the treatment of human disease. The dose of 20 mg. was given two or three times daily to each of 14 hypertensive patients exhibiting increased capillary fragility. Eleven of the fourteen patients were studied for 12 to 16 months and in eight capillary fragility became normal in about eight weeks. Two patients stopped taking rutin after capillary fragility was normal and within six weeks both showed a return of increased capillary fragility. Administration of rutin caused a return to normal within one month. No toxic effects (side actions) were observed. Madison and Pohle[18] gave rutin to 14 cases of purpura (of various origin) and came to the conclusion that certain types of purpura, with *increased capillary fragility, were benefited by such therapy.* Shanno[16] gave 24 each of hypertensive patients 20 mg. of rutin three times daily (a few patients received 40 mg. three times daily). In this group of 24 patients there were thirteen who showed increased capillary fragility, all of whom improved on rutin therapy. Shanno believes rutin is superior to hesperidin or hesperitin because it is a single substance which can be highly purified by repeated crystallization.

Using rats and guinea pigs Wilson et al.[19] performed acute and chronic toxicity experiments with rutin. Rats ingested a diet containing one per cent of rutin for 400 days without showing any histological signs of injury or retardation of growth. Intraperitoneal and intravenous injections of 30 to 50 mg. per kilo given to rats and guinea pigs and 100 to 200 mg. per kilo given to rabbits were innocuous.

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TABLE II

RECOMMENDED DAILY INTAKE OF SIX VITAMINS FOR VARIOUS AGE GROUPS AND THE DIFFERENT PHYSIOLOGICAL CONDITIONS.*

	Calories	Vitamin A, I.U.	Thiamine, mg.	Riboflavin, mg.	Niacin, mg.	Ascorbic Acid, mg.	Vitamin D, I.U.
Man (154 lb., 70 kg.)							
Sedentary	2400	5000	1.2	1.8	12	75	
Physically active	3000	5000	1.5	1.8	15	75	
With heavy work	4500	5000	1.8	1.8	18	75	
Woman (123 lb., 56 kg.)							
Sedentary	2000	5000	1.0	1.5	10	70	
Moderately active	2400	5000	1.2	1.5	12	70	
Very active	3000	5000	1.5	1.5	15	70	
Pregnancy (latter half)	2400	6000	1.5	2.5	15	100	400
Lactation	3000	8000	1.5	3.0	15	150	400
Children up to 12 yrs.							
Under 1 yr.	110/(1 Kg.)	1500	0.4	0.6	4	30	400
1–3 yrs. (27 lb., 12 Kg.)	1200	2000	0.6	0.9	6	35	400
4–6 yrs. (42 lb., 19 kg.)	1600	2500	0.8	1.2	8	50	400
7–9 yrs. (58 lb., 26 kg.)	2000	3500	1.0	1.5	10	60	400
10–12 yrs. (78 lb., 35 kg.)	2500	4500	1.2	1.8	12	75	400
Children over 12 yrs.							
Girls, 13–15 yr. (108 lb., 49 kg.)	2600	5000	1.3	2.0	13	80	400
16–20 yr. (122 lb., 55 kg.)	2400	5000	1.2	1.8	12	80	400
Boys, 13–15 yr. (108 lb., 49 kg.)	3200	5000	1.5	2.0	15	90	400
16–20 yr. (141 lb., 64 kg.)	3800	6000	1.7	2.5	17	100	400

* Nat. Res. Council, Reprint and Circular Series 122, revised 1945.

Summary

In the preceding pages it has been pointed out that several vitamins (substances of diverse chemical nature) are required by man, various animal species and microorganisms for growth and maintenance of normal functions. Most of the vitamins are widely distributed in natural products, some in plant foods, some in animal foods and some in both.

Primitive races lived on wild game, wild berries, seeds and some type of cereal. The cereals were coarsely ground and all parts consumed.

Civilization of man has resulted in a steady refinement of foodstuffs; highly milled flours (65 per cent extraction of wheat) are the rule; vegetables and fruits are picked green and artificially ripened; milk and cream must be pasteurized; these, together with cultivated dislikes, have combined to reduce greatly the daily vitamin intake. The prevalence of several diseases in endemic form (beriberi, pellagra, rickets, scurvy) in certain parts of the world is a reflection of prolonged, nearly complete vitamin deficiencies in the diet of the peoples inhabiting those areas.

The American people, concededly the best fed in the world, should not be subject to nutritional deficiency diseases; however, such diseases do exist in the United States for the reason that the ideal nutritional goal has not been attained. Such a goal can be attained only through the selection of a diet containing all of the required nutrients. In actual practice, however, this ideal is not an easy one for the average person to attain, for the following reasons: first, in the processing of foodstuffs so that they keep well and have a buying appeal, and in the refining of foods and pasteurization of dairy products, considerable (and variable) amounts of the vitamins present in the raw materials are either discarded during preparation by cooking and prolonged holding or destroyed. Secondly, the onward march of civilization has modified the American diet to a considerable extent; our forefathers consumed

greater amounts of meat (wild game), much less sugar, only crudely milled cereals and, although not as varied as they are today, fruits and vegetables in an uncooked state. In recent times there has been an increasing tendency to use greater amounts of carbohydrates with the result that the American people have become the greatest per capita consumers of sugar in the world.

What quantity of each of the several vitamins does man require per day to maintain well-being? How shall man best obtain these essential nutrients when they are lacking in the every day diet? A study of the data presented in Table II, page 244, covering six of the most studied vitamins, will partially answer the first question.

The second problem can be met by supplementing the daily vitamin intake in two practical ways: (a) fortification of staple foods, and (b) making multivitamin preparations a part of the daily diet. Milk and bread have been fortified with vitamin D for several years and recently vitamins A and D have been added to the margarines. In 1941 it was decided to fortify white flour with thiamine (vitamin B_1), riboflavin (B_2), and nicotinic acid (the pellagra-preventive substance) and to label such bread, "Enriched Bread" and also to show the vitamin content per unit of weight.

The few instances of extreme vitamin deficiencies (beriberi, scurvy, etc.) are in no measure indicative of the widespread subclinical conditions, particularly those relating to the factors comprising the B complex, which have been recognized only in recent years. The etiology of these conditions involves more than deficiencies in the diet; it may include any or all of the following diverse factors:

Dietary restriction. Made necessary by surgery, disease (diabetes), obesity and allergy.

Faulty absorption and utilization. Use of colloidal adsorbents (as in treatment of gastric ulcer), diarrhea of long duration, celiac disease, ulcerative colitis, achlorhydria, carcinoma of intestinal tract, liver disease.

Increased excretion. Polyuria (diabetes) or excessive fluid intake ("washing out") and lactation.

Increased metabolism. Fever of long duration, hyperthyroidism, pregnancy, growth, abnormal mental activity (delirium).

The following statement made by Mackie, Eddy and Mills is pertinent at this point:

> "Any pathological condition which interferes with normal food intake, normal digestion, normal absorption of the products of digestion and normal utilization of those products by the liver, may produce secondary or conditioned avitaminosis."

Since June 1942, the labeling of vitamin preparations has included, in addition to the customary units, a statement that the suggested dosage constitutes some integral or fractional part of the minimal daily requirements. In view of the general misconceptions concerning the several vitamin units, this seems to be a simple and desirable method of supplying essential information and may lead to a more intelligent use of vitamin products. Reinforcement of our daily diet with a reasonable amount of multivitamin material may be considered a good investment in health insurance.

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Y

This Book

A Vitamin Digest

By GUY W. CLARK

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